

UNIVERSITÀ DEGLI STUDI DI NAPOLI

“FEDERICO II”

Scuola di Dottorato in Medicina Molecolare

Dottorato di Ricerca in Genetica e Medicina Molecolare



P-TEFb: from AIDS to cell cycle control

Coordinatore:
Prof. Carmelo Bruno Bruni

Candidato:
Dott.ssa Francesca Varrone

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**Dipartimento di Biologia Strutturale e Funzionale , Università di
Napoli Federico II, Complesso di Monte Sant’Angelo.**

Dottorato di Ricerca in Genetica e Medicina Molecolare

Coordinatore Prof. Carmelo Bruno Bruni

**Sede amministrativa:
Dipartimento di Biologia e Patologia Cellulare e Molecolare “Luigi
Califano”**

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**Tesi di Dottorato di Ricerca in Genetica e Medicina Molecolare
XX° ciclo**

P-TEFb: from AIDS to cell cycle control

Canidato: Dott.ssa Francesca Varrone

Docente guida: Prof.Luigi Lania

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1. Introduction

1.1 The transcriptional control by RNA polymerase II

Transcription is a complex process that relies on the collective action of the sequence-specific factors along with the core RNA polymerase II (RNAPII) transcriptional machinery (Figure 1). The control of this process is predominantly mediated by a network of thousand sequence-specific DNA binding transcription factors that interpret the genetic regulatory information and that bind to the proximal promoter and distal transcriptional regulatory regions such as activators and repressors (named enhancers and silencers) (*Kadonaga,2004*). In fact, it has demonstrated that the transcriptional activators recruit the RNAPII-containing transcription initiation apparatus to promoters of protein-coding genes (*Lee and Young 1998*). The binding of RNAPII depends on an associated multi-subunit complex TFIID, which is composed of TBP and TBP-associated factors (TAFs). In particular, TFIID binds TATA via TBP and interacts with regulatory elements through different TAFs. As shown in figure 2, the cis-regulatory DNA elements of eukaryotic is highly structured and exhibits a modular organization consisting of enhancer and silencer and discrete core promoter. It should be considered that exist different protein complexes that interact with these regulatory DNAs. In particular there are three major strategies for regulating the binding and function of RNAPII complex at the core promoter. First, divergent TFIID complexes bind specific sequence elements within the core promoter and recruit RNAPII. Second, multi-subunit transcription complexes that are related to the yeast Mediator complex also facilitate the binding and function of RNAPII. Third, there are enzymatic complexes that remodel or modify chromatin (for example acetylation) (*Levine and Tjian, 2003*).

Target of diverse mammalian activators is the Mediator complex, which is an evolutionary-conserved complex that contains approximately 25–30 subunits and has multiple roles in transcriptional regulation (*Conaway et al.,2005*). Based upon its ability to support activated, but not basal, transcription in vitro, the Mediator complex

is often referred to as a coactivator. However, several Mediator subunits are required for transcription of almost all genes and it has been proposed to be an activator

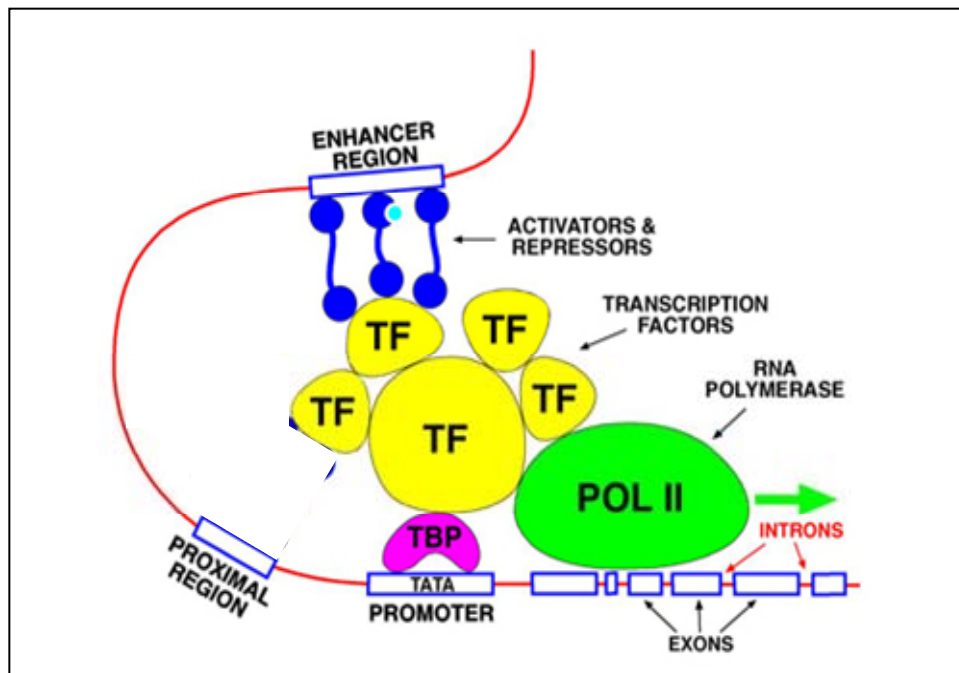


Figure 1: Representation of the eukaryotic promoter and its transcriptional control modules.

The molecular apparatus controlling transcription in human cells consists of three kinds of components: the numbered proteins along with RNA Polymerase II, the basal transcription factors which are essential for transcription and the task falls to regulatory molecules known as activators and repressors. Activators, and possibly repressors, communicate with the basal factors through coactivators-proteins that are linked in a tight complex to the TATA-binding proteins, the first of the basal transcription factors to land on the core promoter.

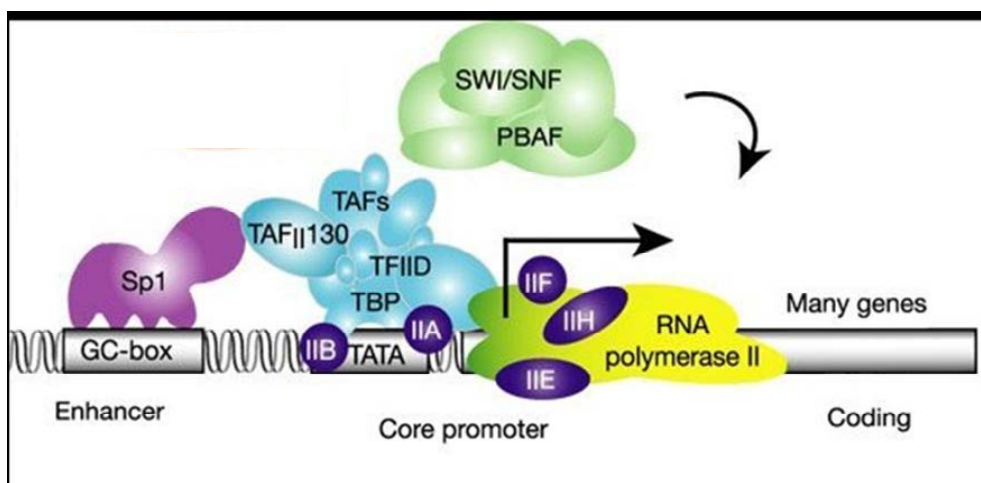


Figure 2: The multi-subunit general apparatus

The eukaryotic transcriptional apparatus can be subdivided into three broad classes of multi-subunit ensembles that include the RNAPII core complex and associated general transcription factors (TFIIA-B-D-E-F-and H), multi-subunit cofactors (Mediator) and various chromatin modifying or remodeling complexes (SWI/SNF, RSF and so on).

target. Several studies have now conclusively shown that distinct subunits of the mammalian Mediator complex are essential and selective targets of different transcriptional activators. This complex is tightly associated with RNAPII molecules that lack phosphate on their Carboxy-Terminal repeat Domain (CTD); in contrast, the elongator complex and various RNA processing factors become associated with RNAPII molecules with hyperphosphorylated CTDs (*Lee and Young, 2000*).

Purified eukaryotic RNAPII typically has 10 to 12 subunits and is capable of DNA-dependent RNA synthesis *in vitro*, but is incapable of specific promoter recognition in the absence of additional factors. It contains, in the largest subunit of RNAPII, a unique Carboxy-Terminal repeat Domain (CTD) that consists of tandem repeats of a consensus heptapeptide sequence (Tyr-Ser-Pro-Tyr-Ser-Pro-Ser). The CTD consensus sequence is highly conserved in eukaryotes, although the number of heptapeptide repeats varies from 26 in *S. Cerevisiae* to 52 in humans. It is known that the functions of the CTD are closely associated with the phosphorylation state of the domain. In fact, the switch in CTD phosphorylation states that occurs between transcription initiation and elongation and it appears to cause the RNAPII molecule to switch cofactor. Moreover, genetic studies have shown that the CTD is essential for the function of RNAPII because deletions that remove only a fraction of the heptapeptide repeats can be lethal (*Nonet, Sweetser and Young, 1987*). Therefore, the RNA polymerase II transcription, could be summarized in a “cycle of events” divided into a number of distinct steps (Figure 3). In order for RNAPII to transcribe a gene, it needs to be recruited to the promoter, assemble with the general transcription factors (GTFs) and then initiate the transcript (pre-initiation complex assembly, open complex formation and initiation). Because regulation of transcription obviously occurs in the context of chromatin and the DNA template is embedded in chromatin, RNAPII transcription thus also entails recruitment of chromatin-remodeling complexes, such as ATP-dependent chromatin remodelling machines and histone-modifying enzymes to facilitate the process. After this, the polymerase needs to escape the ties that bind it to the promoter and become engaged in processive mRNA production (promoter clearance) (*Svejstrup, 2003*). As transcript elongation proceeds, the RNA transcript is matured by capping and splicing, and these events as well as termination-coupled processes leading to m-RNA poly-adenylation all happen co-transcriptionally, that is coupled in the progress of RNAPII through the

gene (*Proudfoot et al., 2002*). Finally, the transcription terminates and RNAPII is recycled for utilization in new rounds of transcription.

The elongation stage of eukaryotic mRNA transcription represents an important regulatory step in the control of gene expression (*Bentley 1995*). Studies during the past several years have demonstrated that the regulation of transcription elongation by RNAPII is not a simple process as once considered to be. Perhaps, the regulation of the RNAPII elongation is a multifaceted and complicated step, as the transcription initiation. The transcription elongation complex, once thought to be composed of merely the DNA template, RNAPII and the nascent RNA transcript, have been studied during the past years have resulted in the discovery of a diverse collection of transcription elongation factors that are either directly involved in the regulation of the rate of the elongating RNAPII or can modulate mRNA processing and transport (*Shilatifard, 2004*). A requirement for phosphorylation of the CTD in elongation by RNAPII was initially proposed on the basis of evidence that CTDs of actively elongating polymerase are highly phosphorylated and that polymerase containing hypophosphorylated CTDs preferentially enter the preinitiation complex, where they are subsequently phosphorylated during or shortly after initiation (*Serizawa, Conaway JW and Conaway RC, 1993*). Phosphorylation of CTD occurs on both Serine2 (Ser2) and Serine5 (Ser5) (Figure 4).

Two kinases have been identified that can phosphorylate the CTD and, based partly their tight association with the initiation apparatus, are almost certainly involved in regulation of transcription initiation. In particular, Ser5 phosphorylation by TFIIF-associated CDK7/cyclinH kinase occurs at or near the promoter, while Ser2 phosphorylation by CDK9-component of P-TEFb elongation factor, is seen primarily on polymerase molecules that have moved away from the promoter region and are engaged in transcript elongation (*Komarnitsky and Buratowski, 2000*). During the transcription cycle concomitant with or following the termination of transcription dephosphorylation of the CTD must occurs in order to regenerate the non-phosphorylated form of the enzyme that appears to be recruited to promoters (*Majello and Napolitano, 2001*). While several RNAPII CTD kinases have been described, has been demonstrated that transcription initiation requires Fcp1/Scp1-mediated dephosphorylation of phospho-CTD. Fcp1 and Scp1 belong to a family of Mg^{2+} -dependent phosphoserine/phosphothreonine-specific phosphatases.

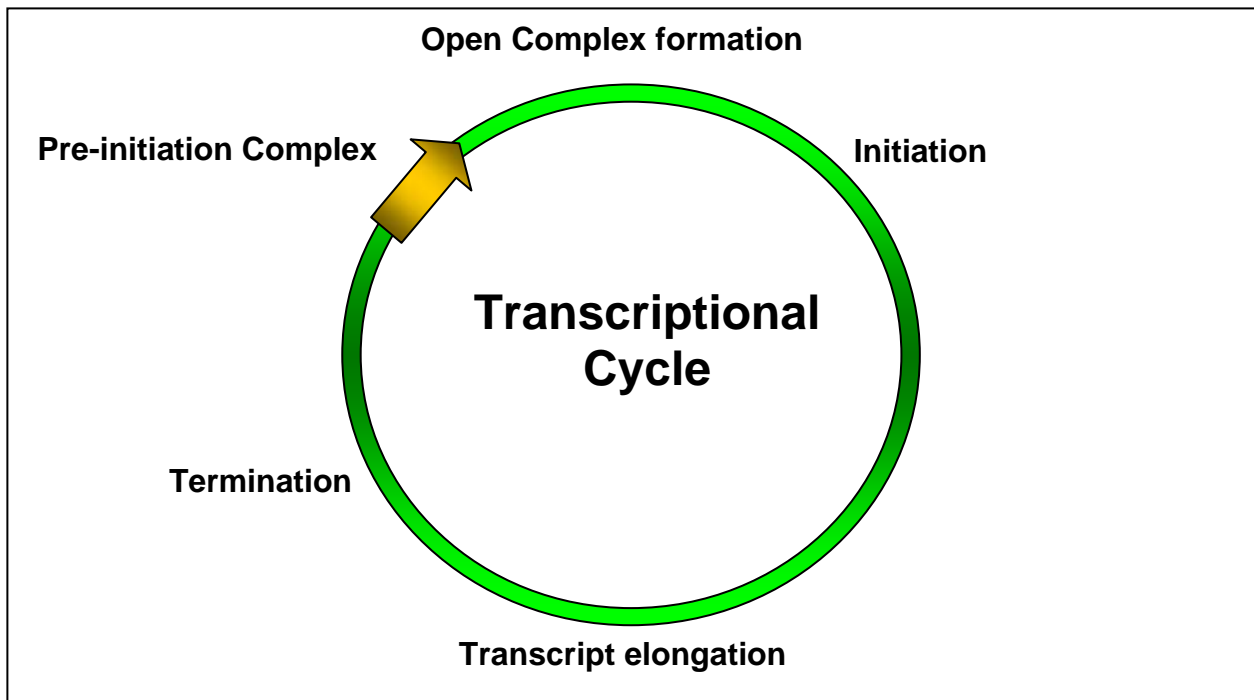


Figure 3: The transcriptional cycle

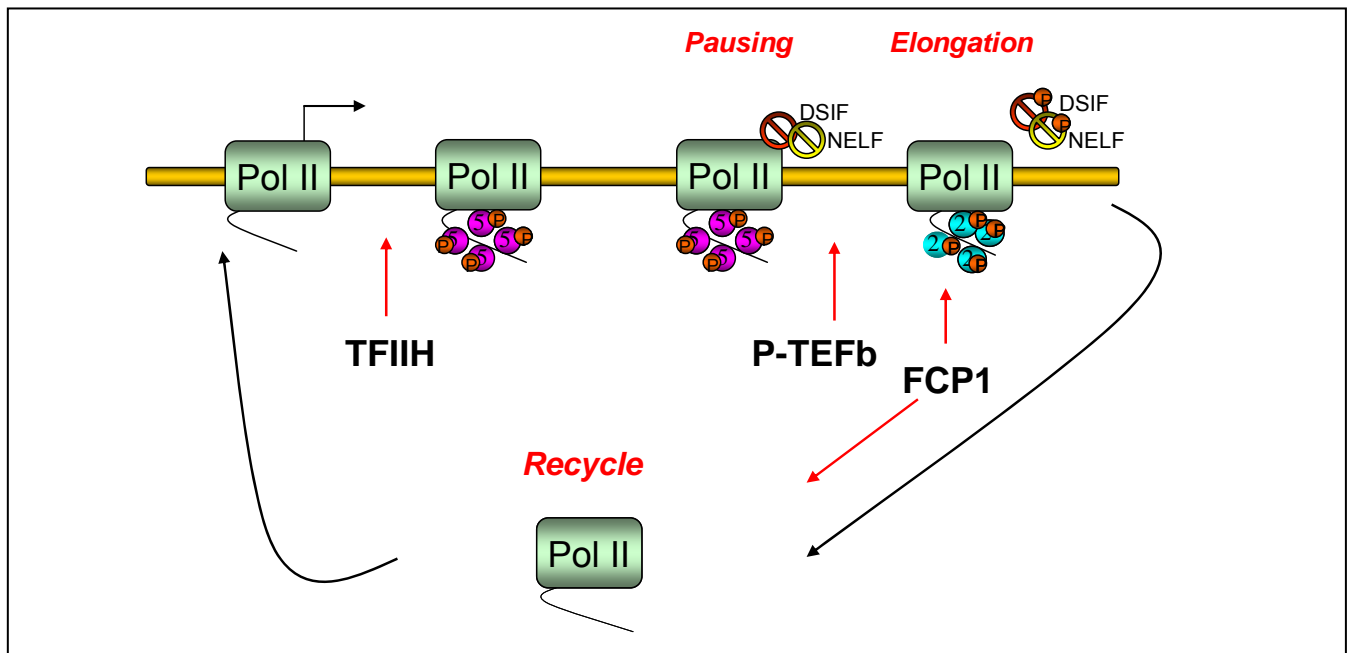


Figure 4: Transcription elongation - The Pol II CTD phosphorylation cycle

After RNAPII has been recruited into a pre-initiation complex, the CTD repeat is phosphorylated on Ser 5 by the **CDK-7** subunit of the GTF TFIID. This phosphorylation is required for Pol II to transcribe beyond the immediate promoter region (clearance), and for recruitment of the mRNA capping enzyme. Subsequently, phosphorylation of CTD Ser 2 by **CDK-9** facilitates elongation and is required for mRNA termination, cleavage, and processing. **CDK-9** is a subunit of the GTF P-TEFb (positive transcription elongation factor b)

Recently *Zhang et al.* showed that Scp1 is an evolutionarily conserved regulator of neuronal discloses the residues in Scp1 involved in CTD binding and its preferential dephosphorylation of P.Ser₅ of the CTD heptad repeat (*Zhang et al., 2006*). Thus it seems reasonable to assume that perturbation of transcript elongation or RNA processing events will also turn out to impact on initiation events at the promoter.

1.2 The P-TEFb complex

Over the past decade, the identity of positive elongation factor (P-TEFb) was revealed. Numerous cellular proteins have been reported to interact with P-TEFb and many studies, demonstrated that about 50% of P-TEFb is present in active form and the other half of P-TEFb exists in a catalytically inactive form (*Nguyen et al., 2001; Yang et al., 2001, Michels et al., 2003*). In particular, the small complex is a heterodimeric complex and is catalytically active and it comprises two subunit, cyclin-dependent kinase-9 (CDK9) and Cyclin T1, T2 or K. In contrast, the large complex is inactive and contains 7SK small nuclear RNA (7SKsnRNA) and HEXIM1 (*Michels et al, 2003; Yik et al, 2003; Chen et al, 2004; Nguyen et al, 2001; Yang et al, 2001*) or HEXIM2 protein (*Blazek et al., 2005*).

Therefore It has demonstrated that P-TEFb is maintained in a functional equilibrium through alternately interacting with its positive and negative regulators (*Jiang et al. 2005*) although the physiological significance of this phenomenon has not been demonstrated clearly. It has shown that active and inactive P-TEFb complexes are in rapid equilibrium, and as shown in figure 5, either a transcriptional arrest, genotoxic insults and UV or RNase treatments, triggers dissociation of 7SK and HEXIM1 from CDK9/Cyclin T resulting in a subsequent accumulation of kinase active P-TEFb complex (*Nguyen et al, 2001; Yang et al, 2001; Michels et al, 2003*). Moreover, it has demonstrated the Hexamethylene bis acetamide (HMBA) induced dissociation of HEXIM1/7SK from P-TEFb; it is also reminiscent of the situations seen with HeLa cells treated with certain stress-inducing agents that globally disrupt transcription and suppress cell growth (*Chen et al.,2004; Michels et al.,2003; Nguyen et al., 2001;Yang et al., 2001; Yik et al., 2003*).Thus, that pharmacological inhibition of P-TEFb kinase activity by 5,6-di-chloro-1-b-D-ribofuranosyl-benzimidazole (DRB) causes a rapid dissociation of 7SK from P-TEFb (*Nguyen et al., 2001 and Michels et*

al.,2003) as well as Actinomycin D that distrupts P-TEFb/HEXIM1 by transcription arrest (*Michels et al.*, 2003).

In particular, HEXIM1 (HMBA-inducible protein 1), is a human protein isolated in smooth muscle cells and it is induced following exposure to hexamethylene bis acetamide (HMBA) (*Ouchida et al*, 2003). It consists of 359 aa and is divided into four regions (as depicted in figure 6): a variable N-terminal region (1–149) that is suggested to have a self inhibitory function; a central nuclear localization signal (NLS, 150–177) that interacts with the nuclear transport machinery and directly binds to 7SK snRNA; a region of highest homology (185–220), including a negatively charged cluster that might be involved in P-TEFb inhibition; and a C-terminal Cyclin T binding domain (TBD) (255–359) that leads to dimerization of HEXIM1 molecules (*Yik et al.*, 2004; *Michels et al.*, 2004; *Shulte et al.*, 2005; *Barbonic et al.*, 2005). It has been reported by *Michels et al.*, 2004 the importance of two domains of HEXIM1 protein, involved in such interactions. Specifically, the HEXIM1 C-terminal domain (181–359) is involved in the binding to P-TEFb through direct interaction with CyclinT1 by the evolutionarily conserved motif (PYNT aa202–205) and the RNA-recognition motif (KHRR) that was identified in the central region of the protein (aa 152–155), involved in a direct binding of 7SK snRNA. Therefore, it has shown that HEXIM1 can form a stable homo and hetero-oligomers with a protein named HEXIM2. HEXIM2 is a protein strongly related to HEXIM1, but smaller, 286 instead of 359 amino acids, and coded by a distinct gene in the same locus (*Dulac et al.*, 2005) which altogether with HEXIM1 may nucleate the formation of the 7SKsn RNP. Despites their similar functions, HEXIM1 and HEXIM2 exhibit distinct expression patterns in various human tissues and established cell lines. Notably, in HEXIM1-knocked down cells, HEXIM2 can functionally and quantitatively compensate for the loss of HEXIM1 to maintain a constant level of the 7SK/HEXIM1-bound P-TEFb. Moreover, it has demonstrated that the HEXIM1 and/or HEXIM2 proteins, in homo- or heterodimeric forms (*Dulac et al.*, 2005; *Yik et al.*, 2005), bind to distal region of 5'-hairpin of 7SKsnRNA (*Egloff et al.*, 2006). Another important component of the inactive P-TEFb complex is the human 7SKsnRNA. It is an abundant, RNA polymeraseIII-syntetized, small nuclear RNA which functions is a key of cellular mRNA production by controlling the activity of the P-TEFb (*Garriga and Grana 2004*). Thus it has reported that docking of 7SK induces a conformational change in HEXIM1 protein that enables their acidic C-

terminal region to interact with CyclinT1 (Michels et al., 2004; Yik et al., 2004; Barboric et al., 2005).

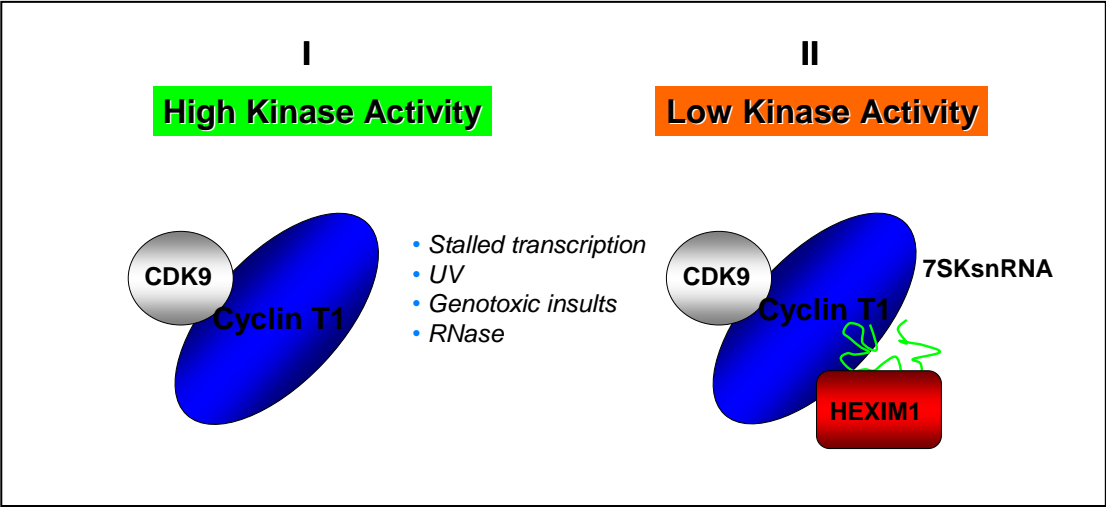


Figure 5: Active and inactive complexes of P-TEFb

P-TEFb is regulated by its reversible association with HEXIM1 and 7SK RNA. When P-TEFb is in this RNA-protein complex, its kinase activity is inhibited.

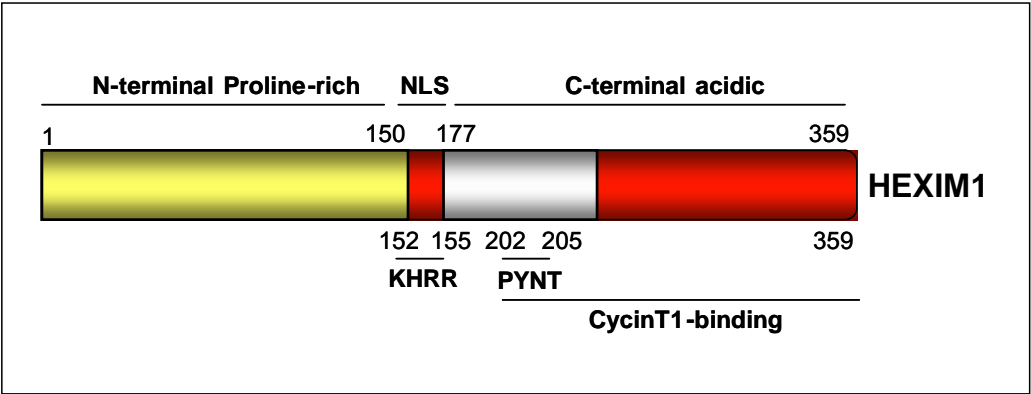


Figure 6: Regulatory domain of HEXIM1 protein

Therefore, recruitment and activation of P-TEFb in vivo, necessitates another RNA-protein interaction formed between the 3'-hairpin of 7SK snRNA and CycT1 (*Egloff et al., 2006*).

Notably, the core active P-TEFb complex, is likely also associated with the positive regulator bromodomain Brd4 (*Jang et al., 2005; Yang et al., 2005*). In fact, besides 7SK and HEXIM1, a bromodomain protein, Brd4 has recently been identified as a major factor associated with CycT1/CDK9 heterodimer (*Jang et al., 2005*). Like all components of 7SK snRNA, Brd4 is also ubiquitously expressed (*Shang et al., 2004*). It belongs to the conserved BET family of proteins that carry two tandem bromodomains and an extra terminal domain (*Yang et al., 2005*). The bromodomain has been recognized as a functional module in helping decipher the histone code through interacting with acetylated histones (*Zeng and Zhou, 2002*). Consistent with this view, Brd4 has been shown to bind to acetylated euchromatin through acetylated histones H3 and H4 (*Dey et al., 2003*). Moreover, the demonstrated binding of Brd4 to CycT1/CDK9 prompted us to investigate a potential role for Brd4 in P-TEFb-dependent transcription. In fact, it has been shown that Brd4 and HEXIM1/7SK existed in two mutually exclusive CycT1/CDK9-containing complexes and stress treatment caused a quantitative conversion of the 7SK snRNA into the complex containing Brd4 bound to CycT1/CDK9 (as shown in figure 7). Importantly, the association with Brd4 contributed to general transcription through its recruitment of P-TEFb to transcriptional templates in vivo and in vitro. Moreover, the P-TEFb complex holds an important role as kinase. It has demonstrated to phosphorylate the C-terminal domain (CTD) of the large subunit (Rpb1) of RNAPII (*Marshall and Price 1995*) capable to play a pivotal role in productive elongation of nascent RNA molecules by RNAPII. In fact, because transcription of pre-mRNAs is blocked shortly after initiation by the DRB sensitivity-inducing factor (DSIF) assisted by the negative elongation factor (NELF) (*Yamaguchi et al, 1999*), the release from this block involves phosphorylation of the carboxyl-terminal domain (CTD) of RNAPII and phosphorylation of negative factors as the Spt5 subunit of DSIF by P-TEFb (*Wada et al, 1998; Ping and Rana, 2001*) which results in a productive transcription. Moreover, components of the inactive P-TEFb complex (7SK RNA and HEXIM1) contribute to the regulation of gene transcription. However, the individual contribution of HEXIM1 and 7SK RNA to the inhibition of P-TEFb kinase activity has remained unclear.

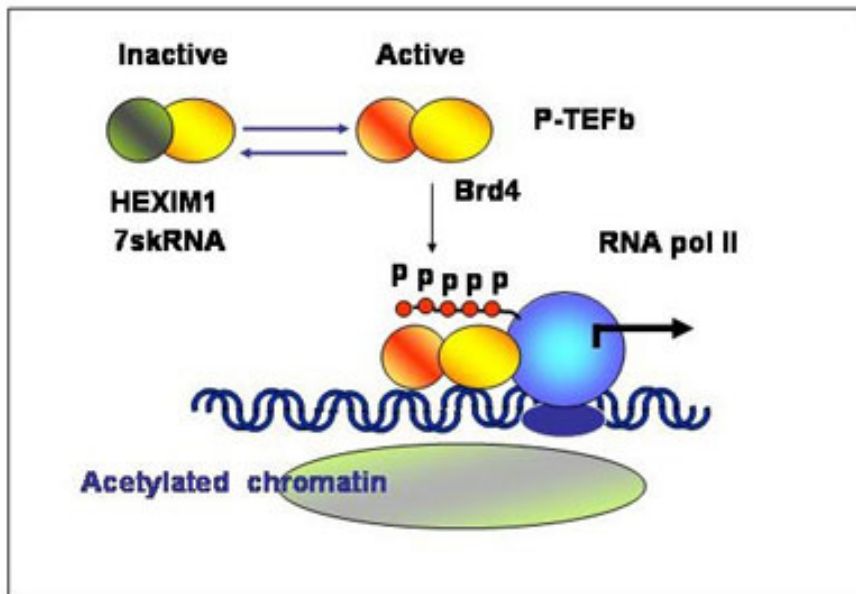


Figure 7: An equilibrium model for Brd4-P/TEFb interactions

P-TEFb occurs either complexed with Brd4 or the inhibitory subunit. The Brd4 bound P-TEFb is recruited to a promoter in acetylated chromatin and stimulates RNA polymerase II dependent transcription

1.3 The role of P-TEFb complex in HIV-1 transcription

Many studies shown that P-TEFb is not only essential for the expression of most protein-encoding genes, but also it is indispensable for the replication of human immunodeficiency virus type 1 (HIV-1) (*Jones et al., 1997; Cullen et al., 1998*).

Transcription of HIV-1 proviral DNA by RNAPII is controlled primarily at the level of elongation by the viral Tat protein (*Barboric and Peterlin 2005*). In fact, many studies revealed that HIV-1 gene transcription and replication require the viral transactivation factor Tat. The important role of Tat protein is manifested on elongating transcription complexes where it alleviates an apparent block to RNAPII processivity at the HIV-1 Long Terminal Repeat promoter (LTR). In absence of Tat, LTR transcripts terminate prematurely: RNAPII clears the HIV-1 promoter but soon arrests due to actions of the negative transcription elongation factor (N-TEF), yielding predominantly short viral transcripts that contain the transactivation response element (TAR) at their 5' ends (Figure 8). Thus, Tat promotes the transition of abortive complexes to processive, elongation-competent complexes, thereby increasing the number of full-length transcripts elongated from the HIV-1 LTR promoter (*Flores et al., 1999*). This is proposed to be necessary to activate transcription elongation from the HIV-1-LTR promoter (*Bienaisz et al., 1998*); in fact, during HIV-1 transcription, P-TEFb travels with the transcription elongation complex as it moves along the HIV-1 transcription unit (*Ping et al., 1999*).

The highly cooperative interactions between Tat, TAR and P-TEFb recruit P-TEFb to the paused transcription complex, where it phosphorylates the C-terminal domain (CTD) of the largest subunit of RNAPII and the components of N-TEF (Negative Transcription Elongation Factor). These events convert N-TEF into a positive elongation factor and recruit pre-m-RNA splicing and 3'polyadenylation machineries to the phosphorylated CTD, resulting in efficient elongation and co-transcriptional processing of nascent pre-m-RNA (*Barboric and Peterlin 2005*). Tat is a protein encoded by HIV-1, transcribed from multiply spliced viral RNA molecules expressed at early stages of viral gene expression. It is composed of the two exons of the viral Tat gene and encodes a protein of approximately 101 amino acids and in the late stage of the infection cycle, a carboxy-terminally truncated, encoded for Tat protein of 72 aminoacids also sufficient to transactivate the HIV-1 promoter.

Although this protein is able to transactivate the HIV-1 promoter in the absence of any viral encoded protein (*Jones et al., 2004*), a considerable body of evidence indicates that Tat interacts with cellular proteins as well as either a highly structured RNA element, transactivation-responsive TAR RNA, which is located at the 5' end of nascent viral transcripts or human CycT1 subunit of P-TEFb, which recruits the kinase complex to the RNAPII elongation machinery (*Bieniasz et al, 1998*) (Figure 9). As shown in figure 10, Tat binds CycT1 by a conserved domain cysteine-rich region, which is part of trans-activating domain. An equally cis-region is essential for direct contact of Tat with TAR RNA cooperatively and induces phosphorylation of the C-terminal domain of RNAPII by CDK9 (*Hetzer et al., 2006*). Tat stimulates transcription elongation through interaction with a transactivation-responsive element (TAR) located at the 5' end of nascent transcripts (*Berkhout et al., 1989*) and it is unique among transcriptional activators in eukaryotic cells in that it functions via RNA rather than DNA promoter elements (*Barboric and Peterlin 2005*). Tat it is the first demonstration of a RNA element of a RNA enhancer element. Neither CycT1 nor the P-TEFb complex binds TAR RNA in the absence of Tat, signifying that binding to RNA is highly cooperative for both Tat and P-TEFb (*Chiu et al., 2004*). Since most of the P-TEFb are sequestered in the catalytically inactive and active complexes in cells, Tat could in principle modulate their configurations to increase the pool of P-TEFb for efficient HIV-1 transcription.

Notably, Tat recruits P-TEFb to the HIV-1 LTR independently of Brd4 (*Yang et al., 2005*). However, it is unclear whether Tat affects the inactive 7SK snRNA. Because it exists an apparent similarities in the molecular RNA protein configurations between the HIV-1 TAR-Tat-P-TEFb and the inactive 7SK snRNA, it has demonstrated that Tat disrupts 7SK snRNA in cells and releases P-TEFb from it via its activation domain. This disruption could be attributed to a direct competition between Tat and HEXIM1 for binding to CycT1. Thus, it appears that HIV-1 has evolved an efficient mechanism that alleviates the negative regulation of P-TEFb by hijacking it from the inactive 7SK snRNP to activate HIV-1 transcription.

HIV-1 proviral DNA is organized into a higher order chromatin structure in vivo, which regulates viral expression by restricting access of the transcriptional machinery to the HIV-1 LTR. The LTR acts as a very strong promoter when analyzed as naked DNA in vitro (*Parada and Roeder, 1996*), while it is almost silent when integrated into the cellular genome. Hence, chromatin conformation essentially represses transcription

from the integrated promoter (*Marzio et al., 1998*). The viral LTR promoter has a structure typical of promoters activated by cellular transcription factors and its “core promoter” which contains three in tandem binding sites for the constitutively expressed Sp1 transcription factor immediately upstream of a TATA box, is necessary for basal levels of LTR-directed RNA synthesis (Figure 9).

Functional analysis of LTR-driven reporter constructs have shown that the mutation of individual or pairs Sp1 sites has a little effect on the basal or Tat-transactivated levels of expression (*Harrich et al., 1990*) but markedly reduces the response of Tat (*Berkhout and Jeang, 1992*). In particular, maximal activation of the LTR requires the concerted action of Tat and cellular proteins binding to the enhancer region, which lies immediately upstream of the core promoter. The region contains two tandemly arranged binding sites (kb-sites) for the dimeric transcription factors composed of several combinations of members of the Rel/NF-kb family of polypeptides (*Liu et al., 1992*). The predominant complex that binds to the LTR-kb sites in activated cells is NF-kb (p50/p65 heterodimer). Furthermore, independent of the viral integration site, five nucleosomes (nuc-0 to nuc-4) are precisely positioned within the 5' LTR as shown in figure 11. In the transcriptionally silent provirus, these nucleosomes define two large nucleosome-free regions spanning nt -255 to -3 and +141 to +265. One nucleosome, nuc-1, is located between these two regions. The first nucleosome-free region in U3 contains many promoter/enhancer elements which are already occupied by transcriptional factors including repressors (*Coull et al., 2002*). Chromosomal integration, an essential step in the viral life cycle, leads to the packaging of the proviral DNA into an array of precisely positioned nucleosomes. These nucleosomes define two open regions of chromatin where transcription factors bind DNA (*Verdin 1991*). Since, as depicted in figure 12, *Kaehlcke et al.*, proposed that Tat also induces chromatin remodelling of a single nucleosome nuc-1 positioned at the HIV-1 promoter stimulating transcriptional elongation of HIV-1 both increasing the intrinsic ability of the RNAPII complex to elongate efficiently and by recruiting histone-modifying enzymes to remodel the elongation block caused by nuc-1 (*Kaehlcke et al., 2003*).

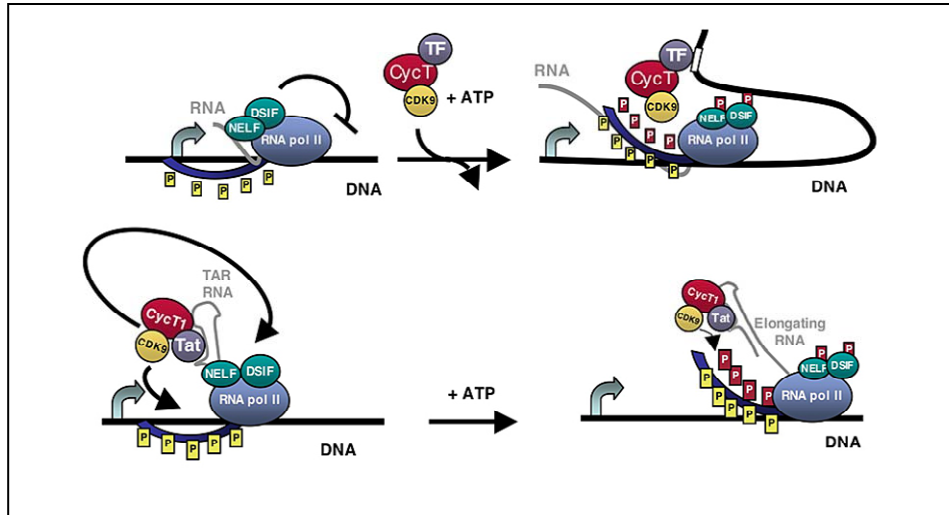


Figure 8: Model for HIV-1 Tat transactivation involving the human P-TEFb complex.

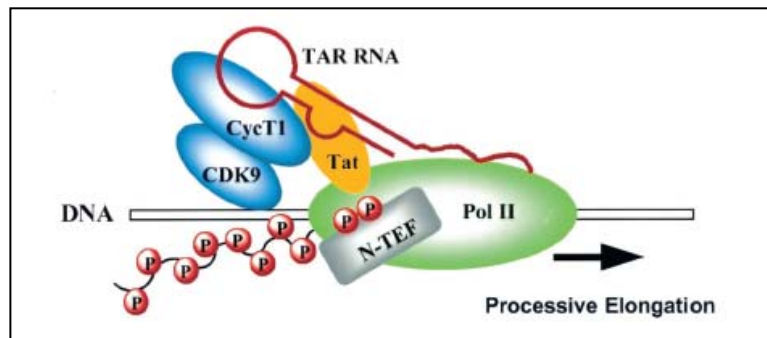


Figure 9: Model for HIV-1 Tat transactivation involving the human P-TEFb complex.

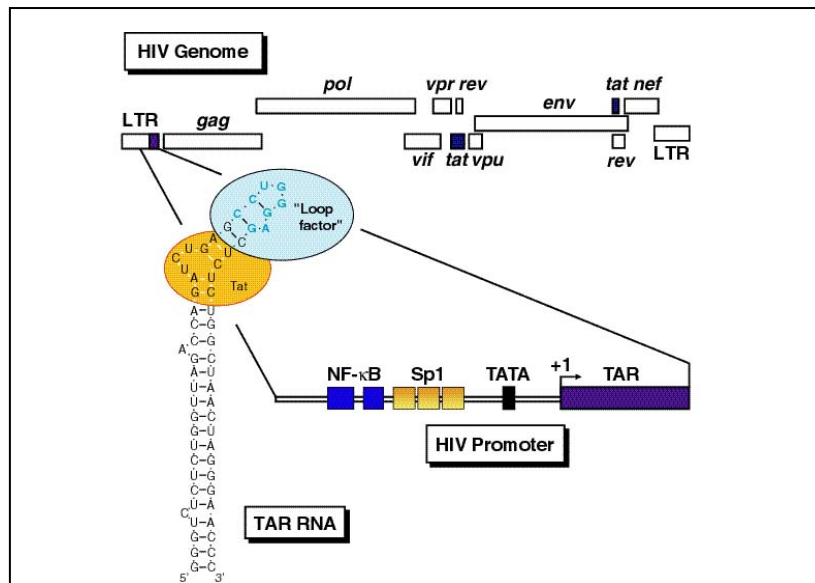


Figure 10: Structure of the HIV genome, the viral promoter and TAR RNA.

The viral promoter has a structure typical of promoters activated by RNA polymerase II. Immediately upstream of the TATA box are two tandem NF- κ B binding sites and three tandem SP-1 binding sites. Immediately downstream of the start of transcription is the transactivation response region (TAR). TAR encodes an RNA that can fold into the stem-loop structure shown at left.

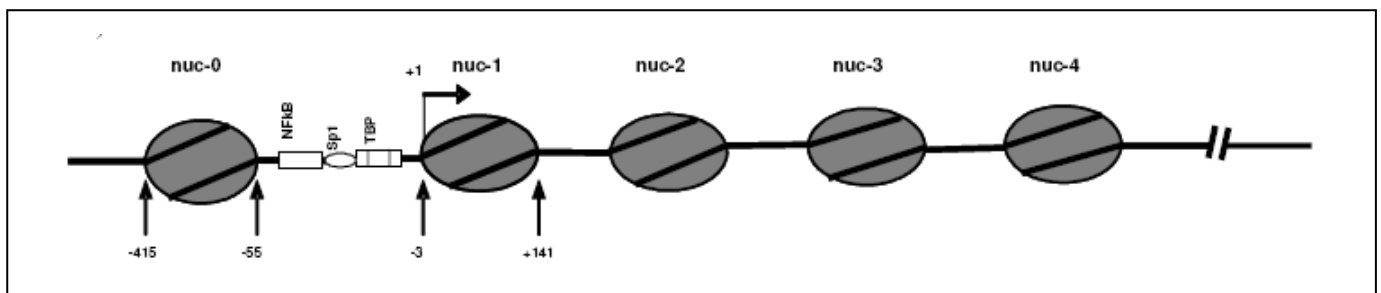


Figure 11: Acetylated Tat associates with a chromatinized HIV-1 promoter near nuc-1

Diagram of nucleosomes positioned on the integrated HIV-1 genome. The transcription start site is indicated as +1. Critical transcription factor binding sites (NF- κ B, Sp1, and TBP) are indicated. Location of nuc-0 through nuc-4 are indicated above the diagram.

1.4 The importance of Acetylation in HIV-1 transcription

The nucleosome remodelling in HIV-1-LTR promoter is thought to remove an obstacle to RNAPII elongation. The molecular mechanism of this Tat-induced nucleosome-remodeling event has remained unclear. Notably it has demonstrated that the chromatin remodeling of nuc-1 that occurs at the HIV-1 promoter in response to Tat, suggests that Tat may recruits also an ATP-dependent chromatin-remodeling complex at HIV-1 LTR to facilitate transcription.

The chromatin remodeling of nuc-1 that occurs at the HIV-1 promoter in response to Tat suggests that Tat may recruit an ATP-dependent chromatin-remodeling complex to the HIV-1-LTR to facilitate transcription . An attractive candidate for the regulation of HIV-1 transcription and chromatin remodelling is a core subunit of SWI/SNF chromatin-remodeling complex (*Wang et al., 1996*). This complex is a component of a class of chromatin-modifying proteins that use the energy from ATP hydrolysis to change the location or conformation of nucleosomes, resulting in increased DNA accessibility within a nucleosomal array. Another group of chromatin modifying complexes are factors that mediate covalent modifications of histones. The N-terminal tails of histone proteins are subject to extensive post-transcriptional modifications, including phosphorylation, methylation and acetylation. The interaction of Tat with a number of histone acetyltransferase (HAT) complexes, such as p300/CBP and PCAF, and their relevance to Tat-mediated activation of the HIV-1 promoter have been established (*Marzio et al., 1998; Kiernan et al., 1999*). Thus, the complexes acetylate the N-terminal tails of histones of nucleosomes at the HIV-1 promoter, inducing destabilization of histone-DNA contacts and facilitating transcription.

Moreover, Tat itself is subject to modification by acetyltransferase (*Ott et al., 1999;; Deng et al., 2000*). In fact, *Mahmoudi et al.* demonstrated that Tat recruits the SWI/SNF complex to the HIV-1 promoter and is necessary for Tat-mediated activation of the HIV-1 promoter (*Mahmoudi et al., 2006*). Other Tat cofactors include a number of transcriptional coactivators with intrinsic histone acetyltransferase activity, including p300/CBP, p300/CBP associated factor (PCAF) and Tip60 (*Barboric et al., 2005*). Notably, chromatin can be remodelled either via the activity of multiprotein chromatin remodelling complexes or the activity of histone

acetyltransferase (HAT) or via the combined activities of both (*Becker and Horz., 2002*). Several transcriptional coactivators with intrinsic HAT activity interact with Tat, including p300/CBP (*Marzio et al., 1998*), Tip60 (*Kamine et al., 1996*) and TAFII250 (*Weiss-man et al., 1998*).

Histone acetyltransferases reversibly catalyze the transfer of acetyl groups on Lysines in the N-terminal tails of histone H2A, H2B, H3 and H4, which form the core of nucleosomes. The level of acetylation of each lysine in the histone tail reflects the competing activities of HATs and histone deacetylases (HDACs) and plays a fundamental role in transcriptional regulation (*Kornberg and Loerch., 1999*). It has reported that the HAT activity of p300 acetylates the Tat protein directly at a highly conserved lysine (K50) in the ARM region (*Kiernan et al., 1999; Ott et al., 1999*). *Kaehlcke et al.*, demonstrated that acetylated Tat interacts with the bromodomain of p300/CBP-PCAF (*Mutjaba et al., 2002*). The interaction between K50-acetylated Tat and PCAF bromodomain is required for Tat transactivation (*Dorr et al., 2002*). Thus, acetylation of K50 occurs as a critical step during HIV-1 transcription that regulates cofactor binding to Tat, both by dissociation of CyclinT1 and association of modification-specific cofactors, such as PCAF (*Kaehlcke et al., 2003*). Therefore, It has suggested that Tat and P-TEFb can also recruit TAF-independent transcription complexes to the HIV LTR (*Raha et al., 2005*). Possibly, this assembly reflects interactions between CycT1 and the unphosphorylated CTD of RNAPIIa (*Taube et al., 2002*). The assembly and disassembly of the complex between PTEFb, Tat, and TAR is a regulated process in vivo. Whereas the phosphorylation of CDK9 strengthens this complex (*Garber et al., 2000*) and the acetylation of the lysine at position 50 in Tat weakens it (*Kiernan et al., 1999*). Upon this disruption, as depicted in figure 12, acetylated Tat is liberated from P-TEFb and recruits the p300/CREB-binding protein– associated factor (P-CAF) to the elongating RNAPII, most likely facilitating chromatin remodeling. Moreover, it has demonstrated that acetylated Tat is deacetylated by SIRT1 (*Pagans et al., 2005*). In this way, Tat can reassemble with P-TEFb on TAR.

Clearly, P-TEFb plays a key role in the control of transcriptional elongation.

Although Tat was the first activator known that could recruit P-TEFb to initiating RNAPII, additional members of this group were soon identified. They include the androgen receptor, c-Myc, the class II transactivator (CIITA), myoblast determination protein (MyoD), and nuclear factor kB (NF-kB). The last one is of great interest as it

explains how the HIV genome can be transcribed before the synthesis of Tat (Barboric et al., 2001). Finally the Tat remodeling of nuc-1 is thought to remove an obstacle to RNAPII elongation. Both Tat activities, P-TEFb recruitment and nuc-1 remodeling, are thought to synergize in enhancing the ability of RNAPII to elongate. The molecular mechanism of this Tat-induced nucleosome-remodelling events has remained unclear (Mahmoudi et al., 2006). In fact, it has demonstrated that the structure of HIV-1 provirus is altered by external stimuli or inhibitors of HDACs activity (Van Lint et al., 1996) which modulate chromatin dynamics (Minucci et al., 2006).

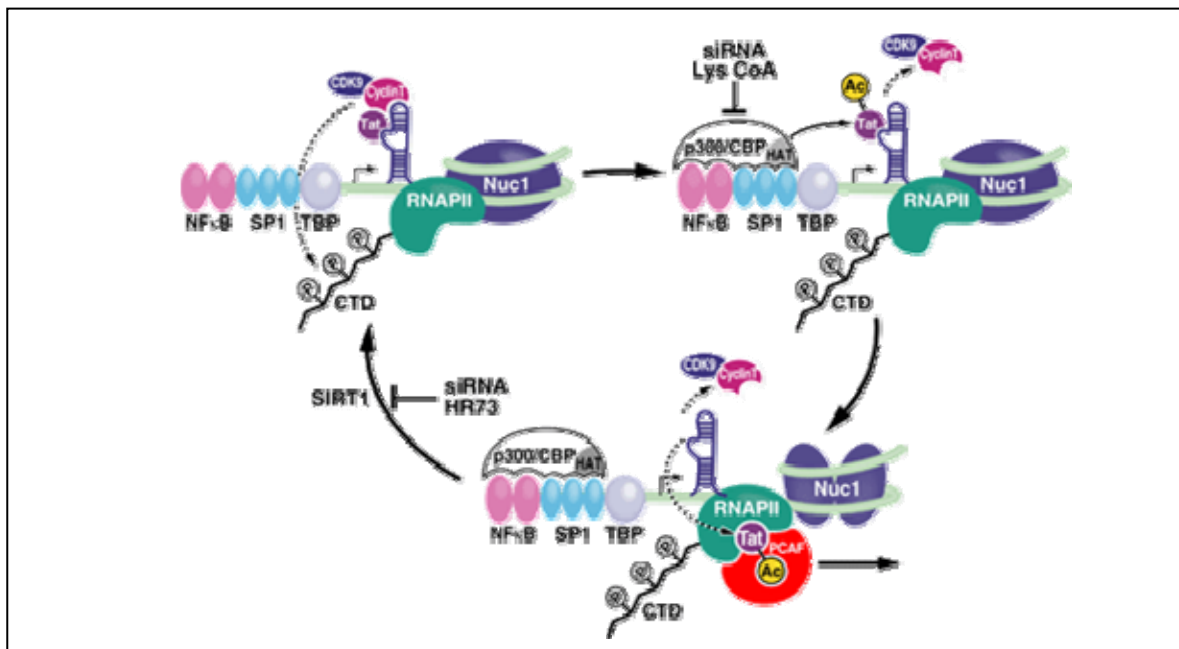


Figure 12: Cycles of Tat acetylation and deacetylation regulate HIV transcription

1.5 The role of HDACs inhibitors in HIV-1 transcription

Nucleosomes the fundamental unit of chromatin structure, provides the first order and, at least in part, the higher-order packaging and compaction of the DNA about 10,000 fold. The nucleosome core particle consists of a highly conserved basic proteins, histone around which 146 bp of DNA are wrapped. Over the past decade, extensive genetic, biochemical and cytological studies have revealed that in addition to their structural role, the histones proteins are also involved in regulation of gene expression. As the maintenance of health depends on the coordinated and tightly regulated expression of genetic information, this becomes a very important function of histones (*Kramer et al., 2001*). Post-translational modifications of histone tails, such as acetylation, phosphorylation and methylation has emerged as common denominators in regulating several biological functions. Acetylation is probably the best understood of these modification reactions. The enzymes involved in this process are Histone acetyl transferases (HATs) and Histone deacetylases (HDACs). Hyperacetylation leads to over expression of a particular gene where as hypoacetylation leads to its repression. Thus HAT and HDAC activity control the level of acetylation and in turn, can regulate the gene expression and its biological functions.

HDACs inhibitors (HDACi) are currently being tested in clinical trials as anti cancer agents (*Marks et al. 2004*). Numerous pharmacological inhibitors of HDACs activity have been identified as given in the classification of HDACs inhibitors. These agents are mainly act by inducing the apoptosis in the cancerous cell. Animal studies have demonstrated that these drugs have little unwanted toxicity and that are an elegant example of how drugs that target cell cycle checkpoints that are defective in tumour cell scan provide the selective toxicity desired in chemotherapeutic agents. HDACs inhibitors are small molecules and restore the acetylation to normal level, induce cell cycle arrest, differentiation, and apoptosis, suggesting their promising anticancer activity (*De Schepper et al., 2003*). A number of small-molecule HDACs inhibitors have been described, showing the capacity to interfere with HDACs activity (*Minucci et al., 2006*). Among then, valproic acid (VPA) has received considerable attention. VPA is an established drug in the long-term therapy of epilepsy, and it has recently reported the treatment of four HIV-positive patients with therapeutic doses of VPA. Infection of CD4 T-cells decreased in all patients (*Lehrman et al., 2005*). The results

of this pilot study suggest that VPA may be a promising addition to HIV-1 treatment. It is currently unknown the molecular mechanism by which VPA reduces the frequency of latently infected, resting CD4T-cells. Most likely, through inhibition of HDACs, VPA allows transcription from silent proviral LTR leading to production of viral proteins and virions and cell death due to virally induced cytotoxicity. However, it is pertinent to note that VPA does not activate resting CD4T-cells, thus making it unclear the molecular mechanisms underlying VPA capacity to activate viral promoter transcription. In my study I demonstrated that HDACs inhibitors such as VPA and Trichostatin-A (TSA) as well as Hexamethylene bisacetamide (HMBA), enhance Tat transactivation capability and activate basal transcription driven by a HIV-LTR promoter. Therefore, TSA is a natural product isolated by *Streptomyces hygroscopicus*, that was initially used as an antifungal antibiotic with the activity of inhibitor of histone deacetylase (*Yoshida et al., 1987*). Consequently, it has been suggested that inhibition of HDACs activity would allow outgrowth of HIV-1 from latently infected resting CD4T-cells. In my works has been showed that HDACs inhibitor such as VPA and TSA as well as the cell differentiation inducer HMBA, enhance Tat transactivation capability and activate basal transcription drive by a HIV-LTR promoter. Although HMBA do not inhibit HDACs (*Ylisastigui et al., 2004*), the shared properties of HMBA and the inhibitors of HDACs to activate HIV-LTR expression are indicative of similarities in their modes of action. In contrast to VPA and TSA, HMBA did not alter the steady-state levels of acetylation, phosphorylation, or methylation at specific sites in the N-termini of core histones (*Ylisastigui et al., 2004*).

It has been suggested that HMBA may non-covalently interact with transcriptional machinery to produce a molecular environment equivalent to that produced by hyperacetylation of histones. HMBA is a hybrid polar compounds that alters factors controlling the G1-S cell cycle phase transition, leading to G1 arrest and inhibition of DNA synthesis. The hybrid polar compounds are potent inducers of differentiation of a wide variety of transformed cells, and HMBA can induce differentiation of neoplastic cells in patients. Taken together, our present knowledge suggests that these agents may be effective in treating cancers (*Marks et al., 1994*). HMBA, a low molecular weight synthetic compound, induces terminal differentiation and apoptosis in transformed cells in culture (*Richon et al., 1996*). Moreover, it has reported that VPA like TSA, inhibits HDACs activity *in vitro* and cause accumulation of hyperacetylated

histone H4 in cultured cells, whereas HMBA do not. Suboptimal antitumour activity at clinically tolerable doses has impeded the further development of HMBA (*Andreef et al., 1992*). HMBA is known to activate HIV expression in chronically infected cell lines (*Antoni et al., 1994*;) and in cell lines stably transfected with long terminal repeat (LTR)-reporter gene constructs (*Zoumpourlis et al., 1992*). While HMBA is structurally related to HDACs inhibitors, it does not inhibit it or induce histone hyperacetylation (*Richon et al., 1998*). Indirect evidence suggests that HMBA and HDACs inhibitors induce cell differentiation by different pathways (*Richon et al., 1996*). Tumour cell lines resistant to the differentiation-inducing activity of HDAC inhibitors are not resistant to HMBA (*Richon et al., 1996*). *Klichon et al.*, find that HMBA increases both initiation and elongation of the HIV-1 LTR in the absence of Tat. Surprisingly, HMBA increased DNA accessibility and induced nucleosome remodelling without histone acetylation. However, unlike mitogen activation, HMBA did not increase cell susceptibility to HIV infection or the expression of cell surface markers of activation. In fact, HMBA down-regulated the surface expression of the HIV receptor, CD4 T-cells. As a likely result of this effect, HMBA did not enhance de novo cell infection, and it suppressed HIV propagation in ex vivo primary blood mononuclear cell (PBMC) cultures. In summary, recent findings suggest that HMBA is a novel prototype for therapeutics designed to interrupt latent HIV infection (*Klichon et al., 2006*). Therefore, a number of small-molecule HDACs inhibitors have been described, showing the capacity to interfere with HDACs activity (*Minucci et al., 2006*).

1.6 P-TEFb and the cell cycle control

Since several years there has been an explosion in the number of publications about the mechanisms that control the cell cycle and how their deregulation can lead to cellular atypia and potentially carcinogenesis. The cell cycle is a ubiquitous, complex process involved in the growth and proliferation of cells, regulation of DNA damage repair and diseases such as cancer. The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and the production of two daughter cells. Central to this process are the cyclin-dependent kinases (cdks) and the cyclin proteins that regulate the cell's progression through the stages of the cell cycle referred to as G1, S, G2 and M phases (Fig.13). The cell cycle can be morphologically subdivided into

interphase and stages of M (mitotic) phase, which include prophase, metaphase, anaphase, and telophase. The G1 and G2 phases of the cycle represented the “gaps” in the cell cycle that occur between the two obvious landmarks, DNA synthesis and mitosis. In the first gap, G1 phase, the cell is preparing for DNA synthesis. S phase cells are synthesizing DNA and therefore have aneuploid DNA content between 2N and 4N. The G2 phase is the second gap in the cell cycle during which the cell prepares for mitosis or M phase. G0 cells are not actively cycling. G0 has since been loosely, and probably incorrectly, used to also include terminally differentiated cells, such as those of the outer layers of the epidermis and adult neurons (*Shafer 1998*).

The core of the cell cycle machinery consists of cyclin dependent kinase (cdk) complexes, the activation of which is strictly regulated during the cell cycle. Each cell cycle phase exhibits a unique pattern of active cdks which conduct the cycle by coordinately phosphorylating their substrates (*Hamel and Hanley-Hide, 1997*). Cdks are partly activated by binding to cyclin subunits, the levels of which oscillate in the course of the cell cycle promoting periodic Cdk-functions. Therefore, inactivation of Cdks takes place partly by phosphorylation of Tyrosine and Threonine residues at the catalytic cleft (*Lew and Kornbluth, 1996*). Additionally, inactivation of Cdks may occur by binding of cdk-inhibitors of the INK4 family (p15, p16, p18 and p19) or the p21 family (p21 Cip1/Waf1, p27kip1 and p57 Kip2) (*Sherr, 1996*).

Notably, cycling cells are continuously monitoring both internal and external conditions and, if required, their cell cycle progression can be halted at so-called cell cycle checkpoints by arresting signals from outside or from inside the cell (*Sherr, 1996*). Therefore, in addition to extracellular signals, changes in the internal milieu and the genome also regulated cell cycle progression (*Sherr, 1996*).

One of the best-known sensors and regulators of the internal cell cycle control pathway is the tumour suppressor p53 whose upregulation after DNA damage leads to growth arrest at least partly via induction of p21 (*El-Deiry et al., 1993*).

p21 was initially characterized as a subunit of Cdk/cyclin/PCNA complexes with an unknown function (*Xiong et al., 1993*). p21 is upregulated by p53 tumour suppressor (*El-Deiry et al., 1993*) as well as to inhibit the activity of various Cdks and to arrest cells in the G1 phase. Although the normal cell signalling machinery may not be functional, the cell must immediately activate the transcription of a subset of genes involved in the individual stress response, while simultaneously repressing numerous

other genes. It has been demonstrated that long before the presence of a cellular stress, cells have already assembled paused RNAPII on the *p21* promoter. In response to DNA damaging agents and p53 activation, the paused, initiating form of RNAPII is converted to its elongating form and transcription of the *p21* gene by RNAPII rapidly ensues (*Espinosa et al. 2003*).

In order to study the mechanisms of p53-dependent transcriptional activation of the *p21* gene, *Gomes et al.* established a high-resolution quantitative ChIP assay that enables to generate detailed maps of protein occupancy on the *p21* locus at different stages of the transactivation process. The gene map in Figure 14 shows the most relevant features of this locus, including the two upstream p53-binding sites (high-affinity BS1 and low-affinity BS2), the transcription start site (+1), the exon–intron organization, the start (ATG) and stop codons (TAA) (*Gomes et al., 2006*). *p21* is expressed at high levels in almost every human tissue examined and independently of the cell cycle stage (*Harper et al., 1993*). Therefore, *p21* levels are regulated during the cell cycle. After initial peak by serum stimulation, *p21* amounts decrease towards the S phase. In addition to G1 arrest, *p21* is known to retard S-phase progression and arrest cells in the G2 phase (*Ogryzko et al., 1996*). It has demonstrate that *p21*, after an initial peak by serum stimulation, it amounts decrease towards the S phase (*Li et al., 1996*) and in most cases it seems to be required for the initial growth arrest but not for the later stages of differentiation. Coinciding with the identification of *p21* as a Cdk inhibitor, *p21* was found to be transcriptionally induced by the p53 tumour suppressor gene product (*El-Deiry et al., 1993*) which is thought to be a central regulator of growth arrest and apoptotic responses after cellular stress and DNA damage (*Levine 1997*). Finally, *p21*, as several genes involved in the DNA damage response pathway contain p53-binding elements upstream of their promoters. In response to chemical or irradiation-induced DNA damage, binding of p53 to target genes occurs rapidly to shut off cell cycle progression and allow for recovery. There are a variety of p53-dependent pathways that can maintain cell cycle blocks (*Agami and Bernards 2000; Gartel and Tyner 2002*). Once *p21* (a potent inhibitor of cyclin-dependent kinases) is expressed, the cells become arrested (*Agami and Bernards 2000*).

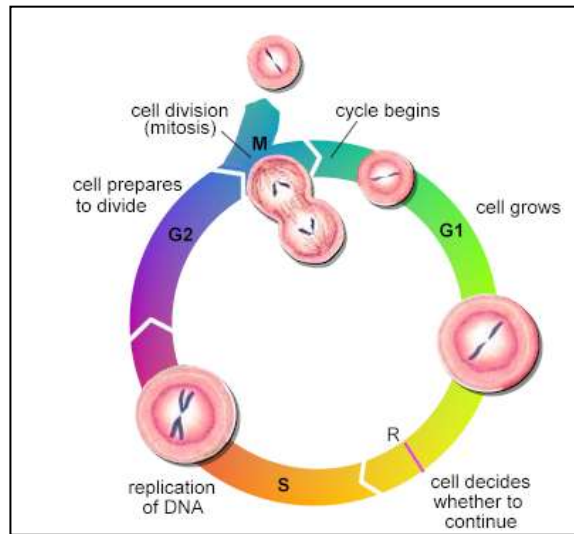


Figure 13: The different phases of the cell cycle.

In the first phase (G1) the cell grows. When it has reached a certain size it enters the phase of DNA-synthesis (S) where the chromosomes are duplicated. During the next phase (G2) the cell prepares itself for division. During mitosis (M) the chromosomes are separated and segregated to the daughter cells, which thereby get exactly the same chromosome set up. The cells are then back in G1 and the cell cycle is completed.

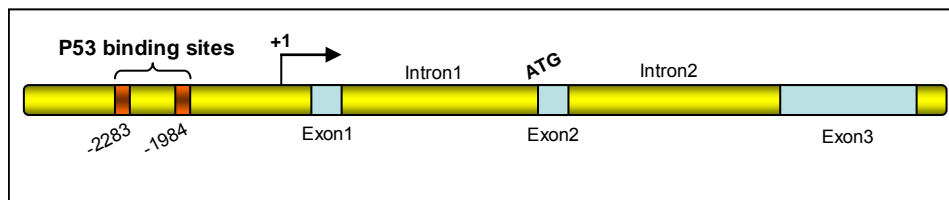


Figure 14: p21 locus

Linear up-to-scale map of the *p21* locus showing the location of p53-binding sites (p3BS1 and p3BS2), the transcription start site (+1), exons and introns, the start codon (ATG), the stop codon (TAA), and the polyadenylation signal (AATAAA).

p53 is one of the most important tumour suppressors in the cell and often referred to as “the guardian of the genome” (Lane 1992). In unstressed cells, p53 is maintained at very low levels. However, also in unstressed cells, some p53 activities involved in

the “routine” maintenance of genomic integrity rely on p53 interactions with genomic DNA, particularly at sites of active metabolic processes that render DNA vulnerable or prone to potential structural re-arrangements (figure 15). In response to various intracellular and extracellular stresses, such as DNA-damage to its integrity, hypoxia and oncoprotein expression, p53 is rapidly stabilized and activated. The transcription activity of p53 is critical to its function such as tumour suppressor, and this is highlighted by the fact that approximately 50% of human cancers contain a mutation of p53 gene. Among them, more than 80% are located in the p53 DNA-binding domain (DBD), which abrogates the p53 transcriptinal activity (*Hainaut et al., 1998*). Therefore, the activated p53 mainly functions as a sequence specific DNA-binding transcription factor to regulate a large number of target genes. These genes mediate cell-cycle arrest, apoptosis, senescence, differentiation, DNA repair, inhibition of metastasis and other p53-dependent activities (*Harms et al.2004*). It has demonstrated that recognition and binding affinity of the various p53 DNA interactions are determined by the presence of specific sequence motifs (sequence-specific DNA binding, p53-SSDB). However, *Gomes et al.* present evidence that CTD kinase activities and RNAPII phosphorylation are differentially required for expression of distinct p53 target genes and that RNAP II is regulated at a post-initiation stage on the *p21* gene. Paused RNAP II assembles on the *p21* promoter before cellular stress and is converted to the elongating form upon stress-induced p53 activation (*Espinosa et al. 2003*). This process is accompanied by recruitment of several positive elongation factors and changes in CTD phosphorylation. Moreover, ChIP assays reveal important differences in the site of recruitment and distribution of the elongation factors NELF, DSIF, P-TEFb, TFIIIF, TFIIH, and FACT (Facilitates Chromatin Transcription) on the *p21* locus.

All these evidence produced several interesting observation: (1) P-TEFb activity is differentially required for activation of distinct p53 target genes; at least two distinct Ser5 kinases act consecutively on the *p21* locus based on DRB sensitivity and site of action; CTD kinases are required for recruitment of the elongation factor FACT to the *p21* locus; (4) P-TEFb kinase activity, Ser2 phosphorylation, and FACT recruitment are dispensable for p21 mRNA transcription, processing, and accumulation in response to stress-induced p53 activation; and (5) global inhibition of transcription triggers a stress response that leads to p53-dependent apoptosis. Thus, the tumour suppressor protein p53 regulates transcriptional programs that control the response

to cellular stress. *Espinosa et al.*, showed that distinct mechanisms exist to activate p53 target genes as revealed by marked differences in affinities and damage-specific recruitment of transcription initiation components. p53 functions in a temporal manner to regulate promoter activity both before and after stress. Before DNA-damage basal levels of p53 are required to assemble a poised RNAPII is converted into an elongating form shortly after stress but before p53 stabilization (*Espinosa et al.*, 2003). In fact it has demonstrated that significant differences in the abundance of poised RNAPII exist at p53 target promoters before activation, in fact, after DNA damage, increased p53 activity leads to the transactivation process by stimulating elongation of arrested RNAPII.

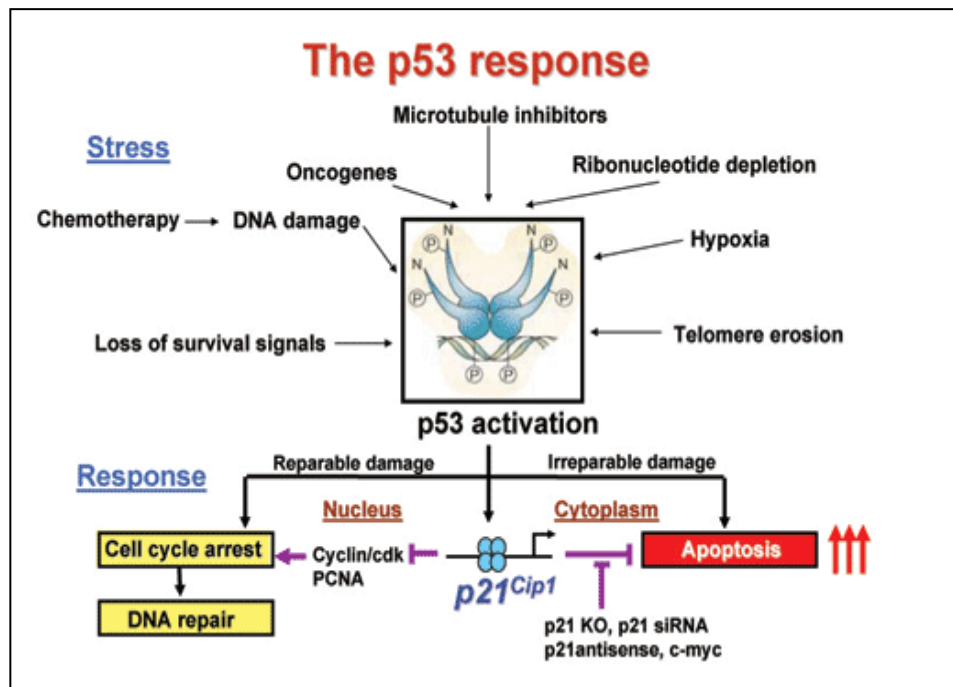


Figure 15:

Summary of the various stress factors that activate the p53 tumor suppressor protein and the opposite functions played by the cdk inhibitor p21 in nucleus and the cytoplasm.

1.7 The cellular stress

In highlight of the studies driven by *Espinosa et al.*, the DRB have an important role on P-TEFb and elongation phase. It has been suggested that this transcription machinery may play an important role in sensing DNA damage and activating DNA repair and stress response pathways when stalled at blocking lesions. There are many different types of DNA lesions that can act on transcription elongation. In fact, it has been shown that the induction of p53 (*Yamaizumi et al.*, 1994) and apoptosis (*Ljungman et al.*, 1996) following UV-irradiation depends on persistent lesions localized in the transcribed strand of active genes. Furthermore, a number of agents that interfere with RNAPII-mediated transcription have been shown to induce p53 and apoptosis suggesting that blockage of transcription may act as a trigger for stress response activation (*Liungman 2005; Ljungman et al. 1999*). It has been shown that UV light induced apoptosis is linked to blockage of transcription (*Ljungman 1999*). Furthermore, treatments with various agents that inhibit RNAPII have been shown to efficiently induce apoptosis in many cell types (*McKay et al.*, 2001), such as two transcription inhibitors named DRB (5,6-dichloro-ribofuranosylbenzimidazole) and ActinomycinD (ActD). These compounds have different mechanisms of action. ActD inhibits transcription but has been reported to inhibit initiation of protein synthesis by interfering with the binding of mRNA to ribosomes (*Singer and Penman 1972*). DRB inhibits synthesis of mRNA by targeting polymerase II transcription (*Yankulov et al. 1995*). In particular, DRB has received a great deal of attention during the last decade and the elucidation of its mechanism of action is of considerable interest. An interesting feature of DRB is its capability to interfere with early transcription events. There is evidence for an effect on transcription initiation as well as for an involvement in premature termination of transcripts. While the biological effects of DRB are extensively studied, there is only limited amount of information available concerning its mode of action and the identification of possible metabolic steps preceding actual transcription block on the chromosomal level (*Egyhazi et al.*, 1999). DRB was originally discovered as an inhibitor of the synthesis of heterogeneous nuclear RNA in human, murine, avian, and insect cells (*Sehgal et al. 1976*). Many of these studies showed that DRB inhibits the synthesis of full-length RNA transcripts by enhancing the pausing or premature termination of transcription by RNAPII. Although it was originally proposed to inhibit transcription initiation (*Sehgal et al. 1976*) specifically at

the level of elongation. DRB-mediated transcription inhibition appears to result from the inhibition of one or more protein kinases necessary for transcription. The other compound involved in transcriptional block is ActD. It is generally thought to intercalate into DNA thereby preventing the progression of RNAPII (Sobell 1985). Moreover, Dubois *et al.* demonstrated that the average phosphorylation of RNAPII-CTD increases in cells exposed to UV, DRB and ActD (Dubois *et al.*, 1994).

Moreover, the cellular stress is also responsive to DNA damage; there are several compounds that induce cellular stress such as Mitomycin C (MMC), Camptothecin (CPT), Apigenin (APG) and Doxorubicin (DXR). In particular, MMC is a DNA alkylating agent that leads to a gradual formation of DSBs in chromosomal DNA, a slowdown of DNA synthesis (Mladenov *et al.*, 2006) and a G2 phase of cell cycle arrest (Franchitto *et al.*, 1998). CPT is a cytotoxic alkaloid with a strong antitumour activity against a wide range of experimental tumours (Gallo *et al.*, 1971). It also inhibits both DNA and RNA synthesis in mammalian cells. The inhibition of RNA synthesis results in shortened RNA chains and is rapidly reversible upon drug removal (Abelson *et al.*, 1972). The inhibition of DNA synthesis, on the other hand, is only partially reversible upon drug removal (Kessel *et al.*, 1972). It is a much stronger inhibitor of DNA synthesis than RNA synthesis (Gallo *et al.*, 1971) and another prominent effect of CPT is the rapid and reversible fragmentation of cellular DNA in cultured mammalian cells (Horwitz *et al.*, 1971). Moreover Flow cytometry (FACS) analyses showed that CPT can induce G1 arrest in cells with normal p53. This G1 arrest was markedly reduced in the p53-deficient cells. These results demonstrate a critical role of p53 as a G1 checkpoint regulator after CPT-induced DNA damage and suggest a rationale for the selectivity of CPT toward tumours with p53 mutations (Gupta *et al.*, 1997). Another candidate agent is APG which have been shown to have antitumour effects in several human adult tumour cell lines, including those derived from various cancers (Casagrande *et al.*, 2001). APG also inhibits UV-induced tumour promotion and the mechanism whereby APG acts, seems to involve p53; it modulates p53 protein levels and accumulation in the nucleus and induce the expression of the p53 target protein p21 (Torkin *et al.*, 2005). Therefore, APG inhibits various cancer cell growth *in vitro* through G2/M phase cell cycle arrest associated with decreased cyclin B-cdc2 activity. Another compound that provokes DNA damage is DXR. It is a compound, similar to DRB, that affects on DNA damage, but it not affects total RNAPII phosphorylation, whereas DRB treatments leads to a clear shift

towards the unphosphorylated form because effectively it blocks CDK9 activity (*Gomes et al., 2007*). One such compound, Caffeine, uncouples cell-cycle progression from the replication and repair of DNA. Caffeine therefore serves as a model compound in establishing the principle that agents that override DNA-damage checkpoints can be used to sensitize cells to the killing effects of genotoxic drugs (*Blasina et al., 1999*). Caffeine may very be the most frequently ingested neuroactive drug in the world. It has been reported that CAF affects cell cycle function, induces programmed cells death or apoptosis and perturbs key regulatory proteins as well as p53 (*He et al., 2003*). Although the effects of caffeine have been investigated, much of the research data regarding caffeine's effect on cell cycle and proliferation seems ambiguous. One important factor may be that CAF has been used in many cell types and under a variety of conditions and concentrations. In fact, it has found that at low concentration (1mM) it induces p53 phosphorylation and p53-dependent apoptosis, whereas at 1-2mM concentration appeared induces G1 arrest and at high concentration (2-5mM) appeared to block G1 arrest and induce apoptosis (*Bode and Dong, 2007*). Previous results demonstrated that CAF overrides the cell cycle effects of various chemicals such as protease inhibitors, preventing apoptosis inhibits cellular DNA repair mechanisms, but notably *Mladenov et al.* has been demonstrated that caffeine is capable to delay and reverse effect of MMC. Finally, a compound named Pifithrin- α , that is a neuroprotective drug based on p53 inhibitors, enhances cell survival after genotoxic stress such as UV irradiation and treatment with Doxorubicin, ectopoxide etc, and a reversible inhibitor of p53 mediated apoptosis and p53-dependent gene transcription as p21 and Mdm2 (*Gudkov et al., 2005*).

Finally, several studies demonstrated that the transcriptional block provoked by cellular stress agents such as DRB, UV and ActD, induces a rapid dissociation of P-TEFb complex. Thus I performed a number of experiments to check if treatments with other compounds that provoke cellular stress by DNA damage and activation of ATM/ATR-p53 pathway, could affect the ratio of P-TEFb complexes. Moreover, I sought to determinate such effect of P-TEFb may represent a general event after the cellular response to genotoxic stress.

2. The aim of this study

An essential factor for regulation of transcriptional gene expression is the positive elongation factor (P-TEFb). Which is present in vivo in two mutually exclusive forms, (*Michels et al, 2003; Yik et al, 2003; Chen et al, 2004; Nguyen et al, 2001; Yang et al, 2001*).

A number of studies have shown that P-TEFb complex is co-factor of the HIV-1 Tat protein. In fact, the Tat activity involves direct interaction with P-TEFb complex (*Garriga and Grana, 2004*) binding to the transactivation response (TAR) element in the nascent HIV-1 transcript (*Price et al., 2000*). In highlight of these studies, in the first part of my thesis, I focalized my attention on the role of the P-TEFb complex in HIV-1 transactivation and the effect of alteration of gene expression, as well as the changes in acetylation state on HIV-1 activation by Tat protein.

In a second part of my studies I focused my attention on the physical and functional interaction between P-TEFb and p53. Finally, the transcriptional block involves a modification in P-TEFb complex equilibrium versus active form, whilst the DNA damage provokes an activation of p53 pathway. I investigated the shift of the P-TEFb complex from inactive to active form upon several stress agents, to understand if P-TEFb activation could be represent an early response to cellular stress and if this effect could be p53-dependent.

3. Results

3.1 Correlation between P-TEFb and HIV-1

3.1.1 Tat activity is inhibited by HEXIM1

The current model for recruitment of P-TEFb to the HIV-1-LTR predicts the formation of Tat-P-TEFb complex, which efficiently binds TAR, allowing CDK9 to phosphorylate the CTD of RNAPII, thereby enhances processivity of the polymerase to produce full-length m-RNAs (*Garriga et al., 2004; Mancebo et al., 1997; Garber et al., 1998*). Tat activity involves direct interaction with P-TEFb complex. However two different P-TEFb complexes exists in vivo in human cells (*Nguyen et al., 2001; Yang et al., 2001*). One is active and restricted to CDK9 and CyclinT1, the other is inactive and contains HEXIM1 or 2 and 7SK snRNA in addition to P-TEFb (*Michels et al., 2004; Yik et al., 2004*). It has shown that Tat interacts only with the active P-TEFb complex (*Michels et al., 2002*). Because the two complexes exchange rapidly, I sought to determine the functional consequences of the over-expression of HEXIM1 and 7SK snRNA on Tat activity. To this end, I performed transient transfection in human 293T cells using HIV-1-LTR-Luc reporter (figure 16 panel A) in the presence of increasing amounts of Flag-tagged HEXIM1 (F:HEXIM1) or 7SK snRNA respectively. I found that Tat-mediated transactivation of the HIV-1-LTR was inhibited by the over expression of F:HEXIM1 in a dose dependent manner; moreover, I found that ectopic expression of 7SK did not significantly affected HIV-1-LTR-Luc basal transcription as well as Tat-activation either alone or in a combination with F:HEXIM1 (figure 16, panel B). As control to test the specific ability of HEXIM1 to repress Tat transactivation, the same experiment was performed using murine CHO cells in which endogenous mouse CycT1 is unable to interact with Tat protein (figure 16, panel C). Finally, like in human cells, it was shown that ectopic expression of 7SK snRNA did not have any significant effect on Tat activity. Notably, the HEXIM1 effect on Tat activity were validated for also its paralog named

HEXIM2 (data not shown and *Fraldi et al., 2005*). These data are consistent with a role of HEXIM1 as negative regulator of HIV-1 activity.

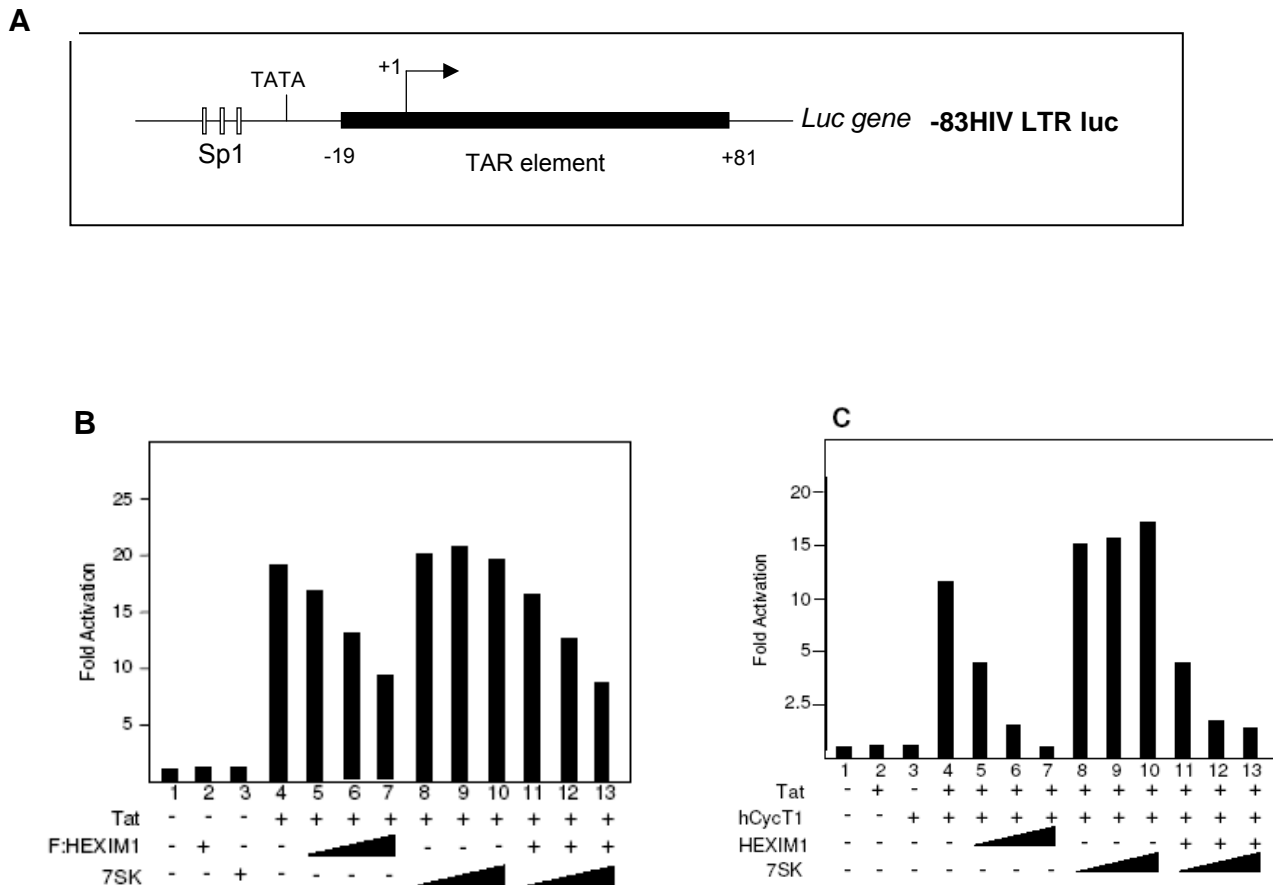


Figure 16 – HEXIM1 is a negative regulator of HIV-1 activity

On top the relevant HIV-LTR luc reporter is depicted (panel A). In the panel B, HIV-Luc reporter was transfected into 293T cells in the presence of pSV-Tat along with increasing amounts of F:HEXIM1 and 7SK RNA as indicated. In the panel C, CHO cells were transfected with HIV-LTRE-luc-reporter in the presence of pSV-Tat and together with CMV-hCycT1, in the presence of increasing amounts of F:HEXIM1 and 7SK. Each histogram bar represents the mean of at least three independent transfections after normalization to Renilla luciferase activity to correct for transfection efficiency with the activity of the reporter without effect set to one.

3.1.2 Definition of the HEXIM1 regulatory domains involved in repression

The following experiments delineated the important structural domains of HEXIM1 required for repression of Tat. It was been reported that the HEXIM1 C-terminal domain (181–359) is involved in the binding to P-TEFb through direct interaction with CyclinT1 (as well as for homo and hetero-dimerization with HEXIM2) and the evolutionarily conserved motif (PYNT aa202–205) is important for such interactions. Notably, it was found that the PYND point mutant is impaired in repression and binding either P-TEFb or 7SK RNA in vivo, albeit it retains the ability to bind 7SK in vitro. In addition, it was determined that HEXIM1 binds 7SK snRNA directly and the RNA-recognition motif (KHRR) was identified in the central region of the protein (aa 152–155). In fact, the HEXIM1-ILAA mutant fails to interact in vivo and in vitro with 7SKsnRNA (*Michels et al., 2004*). It has been demonstrated that the artificial recruitment of P-TEFb to the HIV-1 promoter is sufficient to activate the promoter in absence of Tat (*Majello et al., 1999; Taube et al., 2002*). Consistent with these studies, I addressed the importance of these motifs HEXIM1-mediated repression of Tat activity using a transcriptional system consisting of chimeric Gal4-CycT1 protein and the plasmid reporter G5-83-Luc, which contains five Gal4 DNA binding sites positioned upstream of the HIV-1 long terminal repeat (HIV-1- LTR), followed by the luciferase (Luc) reporter gene. As expected Gal4-CycT1 chimera activates G5-83-Luc in 293T cells, whereas co-expression of the wild-type F: HEXIM1 protein decreased this activity in a dose dependent manner (figure 17, panel A). Similarly, Tat-mediated activation was repressed by HEXIM1 (figure 17, panel B). To confirm the importance of the integrity of HEXIM1 C-terminal domain that would be impaired for P-TEFb binding, I performed the same experiments with point mutants in this domain: the PYNT motif (aa 202–205) in the C-terminal domain that was targeted because it has been conserved throughout evolution from insects to mammals (*Michels et al., 2002*) and PYND mutant in which threonine 205 was replaced by aspartate (*Michels et al., 2004*). I found that, unlike wild-type HEXIM1, both mutants were unable to repress Tat as well as Gal4-CycT1 activities (figure 18, panel A and B), albeit they were expressed at levels comparable to the wild-type protein (figure 18, panel C).

Thus it appears that HEXIM1 inhibition is strictly dependent upon the integrity of the protein to interact with P-TEFb. Moreover, because the point mutant in the central part ILAA abolished HEXIM1 repression on Tat, it can be concluded that HEXIM1-mediated inhibition of Tat required the formation of the P-TEFb/HEXIM1/7SK complex.

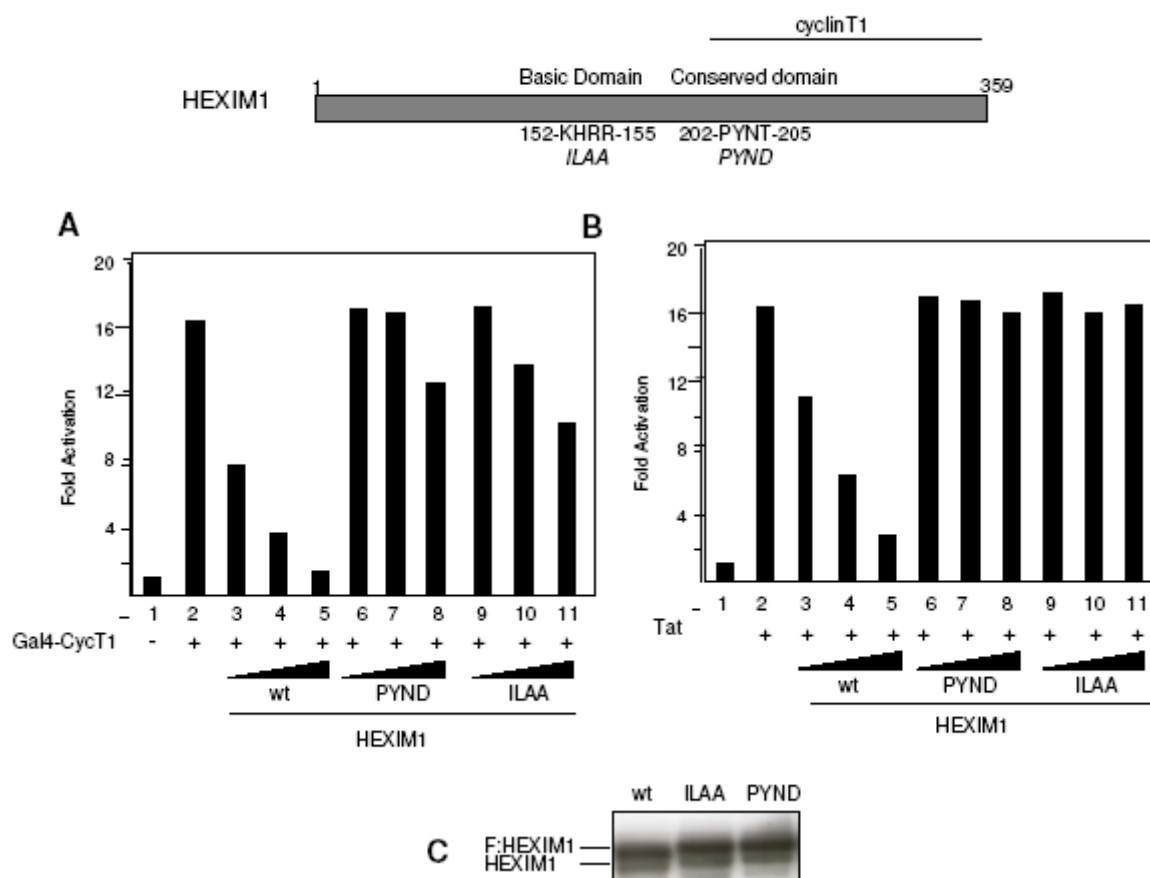


Figure 17 – HEXIM1-mediated inhibition of Tat required the formation of the P-TEFb/HEXIM1/7SK complex.

On top the relevant HEXIM1 functional domains are depicted. Position of the point mutants ILAA and PYND are indicated. G5-HIVLuc reporter was transfected into 293T cells along with Gal4-CycT1. Panel A, or pSV-Tat. Panel B: along with increasing amounts of Flag:HEXIM1 wild type and mutants as indicated. Each histogram bar represents the mean of three independent transfections after normalization to Renilla luciferase activity. Panel C: western-blot with anti-HEXIM1 antibody demonstrated that the HEXIM1 effectors were expressed at comparable levels.

3.1.3 P-TEFb activity in the presence of enhanced expression of HEXIM1

To test whether enhanced expression of HEXIM1 might directly affect the P-TEFb activity, it was performed a time course kinase assay using as a substrate the CTD4 peptide consistent of four repeats of the RNAPII CTD (data not shown and *Fraldi et al., 2005*). It was shown by *Fraldi et al.* that over-expression of HEXIM1 resulted in a modest reduction of P-TEFb activity, thus the inhibition of Tat activity is unlikely due to a global reduction of cellular P-TEFb activity. To further investigate the mechanism of inhibition of Tat mediated transcription by HEXIM1, we tested the relative levels of transfected Tat protein in the presence of F:HEXIM1. I found that ectopic expression of HEXIM1 did not affect Tat expression (figure 18, panel A). Next, I sought to determine whether exogenous expression of HEXIM1 might result in a decrease in Tat-bound CycT1. To this end 293T cells were transfected with pSV-Tat in the presence or absence of F:HEXIM1 using the same transfection conditions used in the Luciferase assays. Cells extracts were immunoprecipitated with CycT1 antibody and the immunoprecipitates were analyzed by immunoblotting for evaluation of Tat, CycT1 and HEXIM1 proteins, respectively. In two different experiments I found a modest, but reproducible decrease in Tat-bound cyclin T1 (fig. 18, panel B).

Collectively, these data demonstrated that over-expression of HEXIM1 resulted in a modest reduction of P-TEFb activity, thus the inhibition of Tat activity is unlikely due to a global reduction of cellular P-TEFb activity, rather over-expression of HEXIM1 appears to influence Tat-P-TEFb interaction.

3.1.4 Activation of HIV-1 LTR promoter by HDACs inhibitors and HMBA

The integrated HIV-1 provirus is assembled into an ordered chromatin structure altered by external stimuli or inhibitors of HDAC activity (*Van Lint et al., 1996; Williams et al., 2006*). One of the most important mechanisms responsible for chromatin remodeling is the post-translational modifications of histone tails. Histones are subject to dynamic covalent modifications that modify gene expression, including acetylation, methylation, ubiquitylation, and ADP-ribosylation. Dynamic changes in gene expression may affect chromatin structure and, consequently, the interaction of chromatin with regulatory factors. A class of compounds that modulate chromatin dynamics are the HDACs inhibitors. Several studies suggested that treatment of cells with the antiseizure

drug valproic acid (VPA, 2-propylpentanoic acid) results in histone hyperacetylation, growth arrest, and cell differentiation in several tumor cell lines (Minucci *et al.*, 2006).

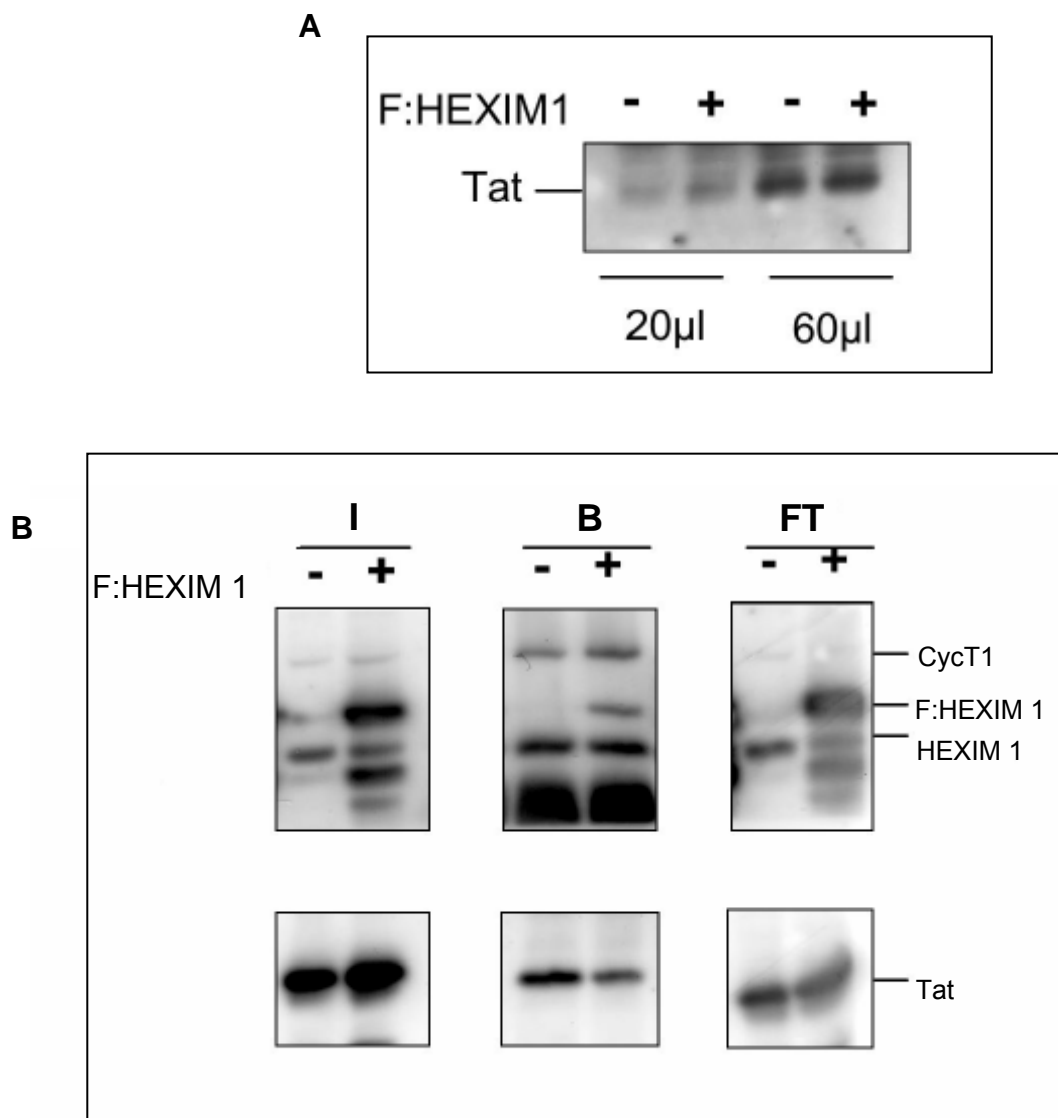


Figure 18 – Tat- CyclinT1 binding in the presence of HEXIM1.

Tat-CyclinT1 binding in the presence of HEXIM1. Panel A: 293T cells were transfected with pSV-Tat in the presence or absence of F:HEXIM1 as indicated and at 48 hrs after transfection cell extracts were probed by Western blotting with anti-Tat. For accurate comparison increasing amounts of material (µl) were loaded on the gels. Panel B: 293T cells were transfected as in panel A, and cell extracts were immunoprecipitated with anti-CyclinT1. Immunocomplexes were analyzed on Western blots as indicated. I, input; B, bound; FT, flow through. This experiment was performed two times with similar results.

To investigate the effect of VPA on the expression of a reporter gene under the control of a defined HIV-1 promoter, I performed firefly Luciferase assay that

was followed in lysates from huHL6 cells exposed to VPA or HMBA, in the presence or absence of pcDNA3-Tat-101-flag, respectively. huHL6 cells is a clonal human cell line bearing an integrated HIV-1-LTR-Luc reporter derived from HeLa cells co-transfected with pHIV-1LucA41 and pRSVtkneo. I found that both VPA and HMBA activates basal as well Tat-mediated HIV-1-LTR expression (fig 19, panel A). To further substantiate these findings I also tested the effect of HMBA and the HDACs inhibitors (VPA and TSA) in transient transfection assays. To this end, I transfected human 293T cells with –83HIV-1-Luc reporter along with pcDNA3-Tat101 in the presence or absence of TSA, VPA and HMBA, respectively. The –83HIV-1-Luc reporter contains the LTR sequences spanning from –83 to +82, thus bearing the three Sp1 binding sites, the TATA element and TAR sequences. As shown in figure 19 panel B, both HMBA, TSA and VPA induced a robust increase of basal and Tat-activated expression of the –83HIV-1-Luc reporter in transient transfection. Because Tat activity is mainly dependent upon the interaction with catalytic active P-TEFb (*Brady et al., 2005*), I monitored the relative amounts of P-TEFb (CDK9 and CycT1 proteins) as well as the P-TEFb-bound inhibitor HEXIM1 upon VPA, TSA and HMBA treatment, respectively. As reported in figure 19, panel C, the relative amounts of CDK9 and CyCT1 proteins remained largely unaffected. In contrast, treatments with HMBA as well as in the presence of HDACs inhibitors a slightly enhancement of the HEXIM1 protein levels were observed.

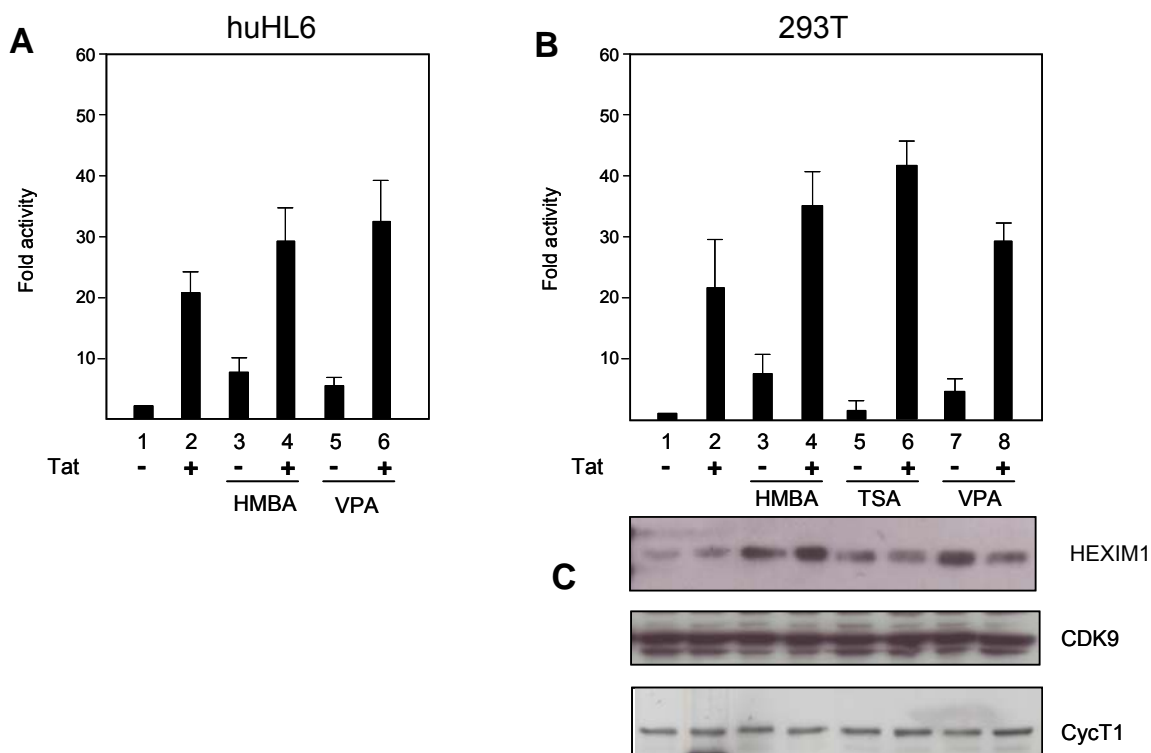


Figure 19 – Enhancement of HEXIM1 as a result of HMBA and HDACs inhibitors.

HuHL6 cells harboring an integrated HIV-LTR-Luc reporter were transfected with pcDNA3-Tat vector as indicated, and after 24 h mock and Tat-transfected cells were treated with VPA and HMBA for additional 24 hr. Cell lysates then prepared were analyzed for luciferase activities. Each histogram bar represents the mean of at least three independent transfections with the activity of the untransfected huHL6 cells without Tat without effect set to one. (B). Human 293T cells transfected with –83HIV-Luc reporter in the presence or absence of co-transfected pcDNA-Tat vector as indicated. Cells were then were treated (24 hr after transfection) with VPA, TSA and HMBA for additional 24 hr. Cell lysates then prepared were analyzed for luciferase activities and immunoblottings with the indicated antibodies (panel C). Each histogram bar represents the mean of at least three independent transfections with the activity –83HIV-Luc reporter without Tat vector set to one.

3.1.5 HEXIM1 represses VPA and HMBA activation of HIV-1-LTR promoter

HMBA-treatment of virtually any cell line tested results in induction of HEXIM1 protein (*Yik et al., 2005; Turano et al., 2006*). However, induction of HEXIM1 is not restricted to HMBA treatment. Rather, it has recently found that HEXIM1 is up-regulated by various chemical inducers, such as Retinoic acid and DMSO (*Turano et al., 2006*) as well as HDACs inhibitors (VPA and TSA) as reported in figure 19 panel C.

Paradoxically, high levels of Tat transactivation were detected in cells treated by HMBA and VPA, albeit a modest increase of endogenous HEXIM1 protein was seen. Subsequently, I sought to determine the effect of over-expression of HEXIM1 protein on the HIV-1 promoter activity in the presence of HDACs inhibitors and HMBA respectively. I co-transfected Flag-tagged HEXIM1 expression vector (F:HEXIM1) into 293T cells together with –83HIV-1-Luc reporter (*Majello et al., 1999, 2005*) along with pcDNA3-Tat-101-flag and transfected cells were then treated with the HDACs inhibitors and HMBA, respectively. I found that over-expression of exogenous F:HEXIM1 inhibits the stimulatory effect exerted by the HDACs inhibitors (VPA or TSA) as well as by HMBA treatment (fig. 20 panel A and B).

Because both Tat and VPA stimulate basal transcription, the inhibitory function of F-HEXIM1 might be due to interfering with Tat function and/or to inhibition of VPA action. To address this point I determined the functional consequences of F:HEXIM1 expression in the absence of Tat. 293T cells were co-transfected with –83HIV-1-Luc reporter along with F:HEXIM1 and luciferase activity was followed in lysates from cells exposed to VPA and HMBA, respectively. As shown in figure 21 (panels A and B), F:HEXIM1 effectively debilitates the activity of HDACs inhibitors in a Tat-independent manner.

The identification of HEXIM1 as a P-TEFb-associated factor and P-TEFb as a principal target of HEXIM1 (*Yik et al., 2004; Michels et al., 2004*) suggest that P-TEFb plays a key role in HIV-1 LTR activation mediated by HDACs inhibitors in the absence of Tat. To further substantiate the role of P-TEFb in VPA-induced activation, I carried out co-transfection assays using another specific inhibitor of P-TEFb activity. It has previously described an effective

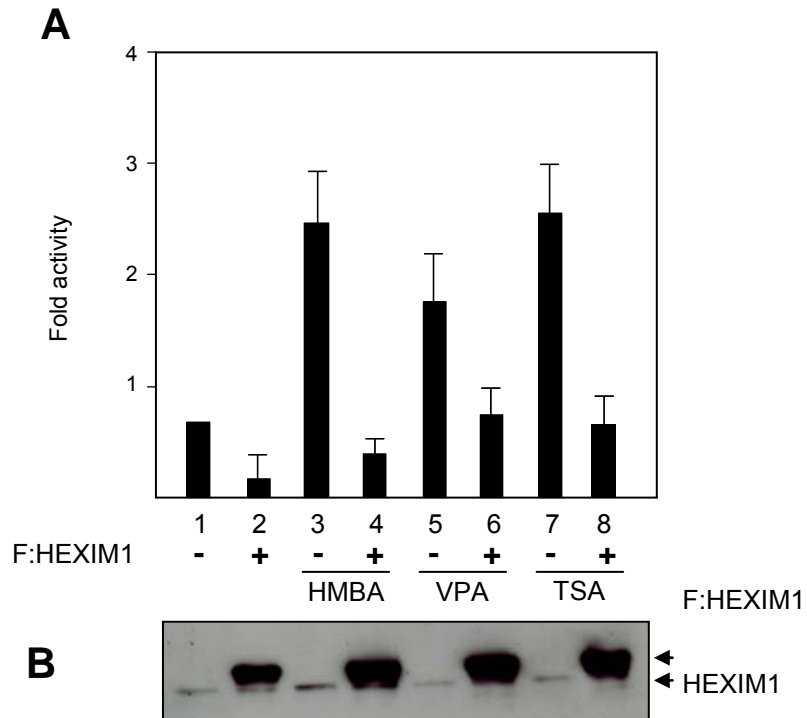


Figure 20 – Over-expression of F:HEXIM1 inhibits the stimulatory effect of HMBA and HDACS inhibitors

293T cells were co-transfected with –83HIV-Luc reporter together with pcDNA3-Tat vector , in the presence or absence of F:HEXIM1 vector as indicated. After 24 hr cells were treated with VPA, TSA and HMBA for additional 24 hr as indicated. Cell lysates were then prepared and analyzed for luciferase activities (A) and immunoblotting with the anti-HEXIM1 antibody (B). Each histogram bar represents the mean of at least three independent transfections with the activity of the cells transfected with Tat alone set to one.

protocol to achieve functional ablation of CDK9 in vivo using a strategy named oligomerization protein inactivation (OCR). This strategy relies on the ability of the coiled-coil (CC) region of the nuclear factor promyelocytic leukemia (PML) to form self-associating oligomeric complexes (*Contegno et al., 2002*). It was demonstrated that fusion of “CC domain” to CDK9 triggers the formation of large molecular weight hetero-complexes leading to a functional inactivation of the CDK9/Cyclin T1 transcription regulatory properties (*Napolitano et al., 2003*). 293T cells were transfected with –83HIV-1-Luc reporter in the presence or absence of flag-cc-CDK9, then transfected cells were treated with VPA and HMBA, respectively. We determined that expression of cc-CDK9 molecules effectively inhibits VPA as well as HMBA activities (figure 21 panel C and D).

Collectively, the data presented in figures 19, 20, 21, strongly suggest the crucial role of P-TEFb in HMBA or VPA induced activation of HIV-1-LTR promoter in the absence of Tat.

Several studies have highlighted the role of cis-acting DNA elements within the Long Terminal Repeat (LTR) in Tat transactivation. Although Tat stimulates mainly chain elongation, it requires cellular DNA-bound activators to stimulate transcription efficiently. In fact, mutation of cis-acting regulatory elements within HIV-1-LTR, such as Sp1 DNA-binding sites, strongly affect Tat transactivation (*Cullen et al., 1993; Jones et al., 1994; Southgate et al., 1991*). To monitor the role of cis-acting sequences in HMBA or VPA activation of HIV-1-LTR expression, I performed the luciferase assay in 293T cells transfected with three different HIV-1-LTR-Luc reporters: G5-83-Luc, in which Gal4-binding domain is fused to wild-type HIV-1-LTR inserted upstream to luciferase gene, G5-38 in which HIV-1-LTR have the Sp1 binding sites deleted and finally G5-83 Δ TAR-Luc that was made deleting TAR region (*Majello et al., 1998*). I transfected 293T cells with these reporters (which structure is depicted in figure 22 panel A) and I exposed these cells to HMBA and VPA treatments for 24 hours. I found that both HMBA and VPA activates all three reporters either wild-type than LTR mutants, albeit the relative basal levels of each reporter were significantly different. Thus, HMBA and VPA activate basal transcription in a TAR-independent manner.

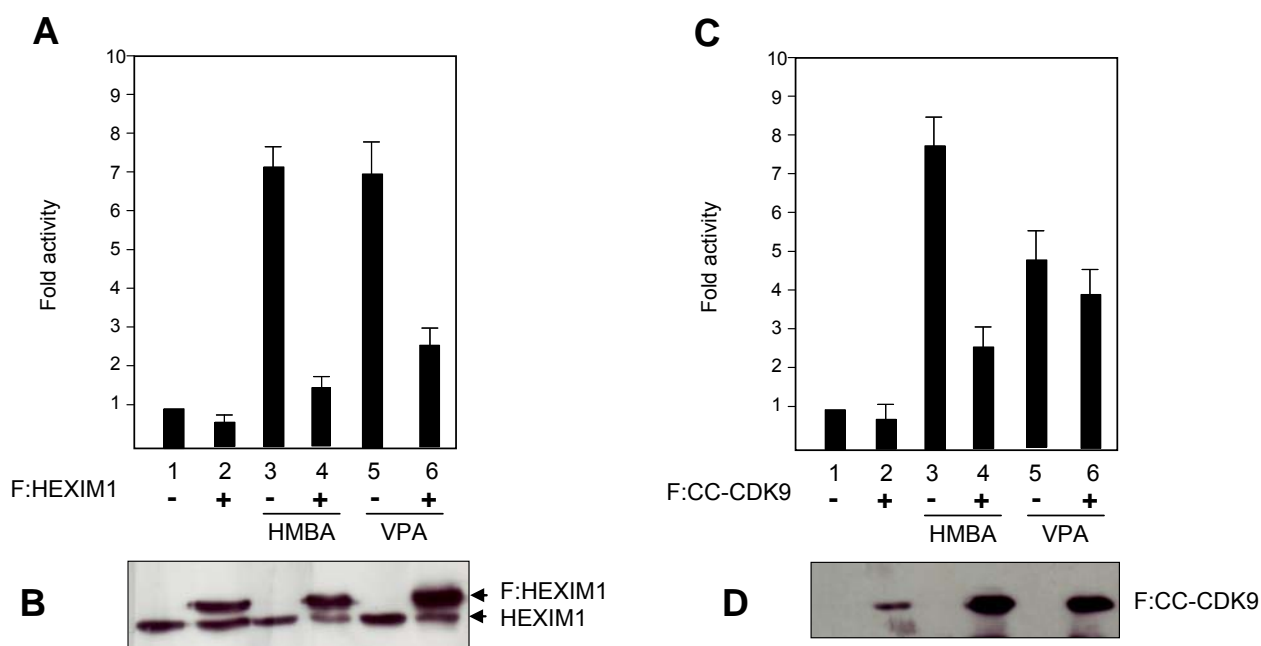


Figure 21 – HEXIM1 debilitates the activity of HDACs inhibitors in a Tat independent manner.

293T cells were transfected with -83HIV-Luc reporter in the presence or absence of F:HEXIM1 (panel A) or FLAG-CC-CDK9 (panel C) as indicated. After 24 hr cells were treated with VPA, and HMBA. Cell lysates then prepared and analyzed for luciferase activities (panels A and C) and immunoblotting with the anti-HEXIM1 antibody (B) or anti-FLAG (D). Each histogram bar represents the mean of at least three independent transfections with the activity of the cells transfected with -83HIV-Luc reporter set to one.

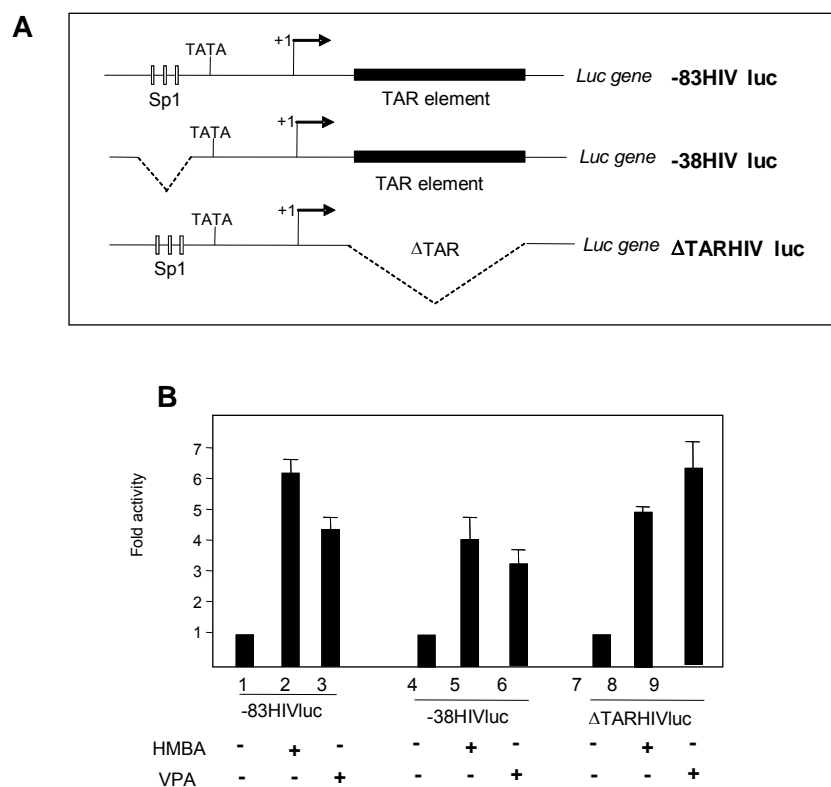


Figure 22 – HIV1-LTR wild-type and mutants in response to HMBA and HDACs inhibitors treatment.

On top the HIV-LTR reporter mutants are depicted (A). HIV-Luc reporter mutants was transiently transfected into 293T cells, 24 hr after transfection cells treated with VPA and HMBA (B). 293T cells were transiently co-transfected with HIV-Luc reporter and PCDNA3-tat-101-flac; 24 hr after transfection cells treated with VPA and HMBA.

3.1.6 VPA induces recruitment of P-TEFb in the absence of Tat

Chromatin structure is modulated by the covalent modifications of the N-termini of the core histones in nucleosomes and by the action of ATP-dependent chromatin remodeling complexes. In particular, histone acetylation at the promoter of genes, mediated by histone acetyltransferases (HAT), has been shown to be necessary, albeit not sufficient, for transcriptional activation (*Berger et al., 2002;; Narlikar et al., 2002*). Each HAT has its own lysine specificity within the tails of histones H3 and H4, leading to the notion of a 'histone code' that determines the epigenetic control of transcription (*Strahl and Allis, 2000*). Experiments performed both in vivo (*Verdin et al., 1993; Van Lint et al., 1996; El Kharroubi et al., 1998*) and in vitro using the HIV-1 promoter reconstituted into chromatin (*Van Lint et al., 1996; El Kharroubi et al., 1998*) have shown that, independent from the integration site, nucleosomes in the 5' LTR are precisely positioned with respect to cis-acting regulatory elements. In the transcriptionally silent provirus, these nucleosomes define two large nucleosome-free areas. The first one is composed of the core promoter, containing three tandem Sp-1 binding sites and the TATA box sequence, and of the LTR enhancer, which is the target for the p50/p65 NF- κ B heterodimer; the second open area spans the primer binding site immediately downstream of the 5' LTR. These two open regions are separated by a single nucleosome called nuc-1 that is specifically and rapidly destabilized during transcriptional activation. The position of nuc-1 in the close proximity of the transcription start site and its displacement during transcriptional activation suggest that chromatin plays a crucial role in the suppression of HIV-1 transcription during latency and that nuc-1 disruption is necessary for transcriptional activation (*Verdin et al., 1993; Van Lint 1996*). The Tat mediated recruitment of HAT proteins most likely explains the changes in chromatin conformation observed at the LTR upon transactivation (*Verdin et al., 1993; Van Lint et al., 1996*). Moreover, changes in chromatin conformation were also upon treatment with HDACs inhibitors (*Wade 2001*).

Based on these considerations I sought to determine the presence of histone acetylation as well as presence of RNA-polymerase II and P-TEFb on HIV-1-LTR in the presence of HMBA and VPA, respectively. To this end 293T cells were transfected with -83-HIV-1-Luc reporter and cells were treated for 24 hours with HMBA and VPA. As expected I found activation of transcriptional activity on HIV-1-promoter (figure 23, panel A). In

parallel, I carried out the powerful approach of chromatin immunoprecipitation (chIP) to map the presence of specific transcription factors at HIV-1-LTR promoter in the presence or absence of HMBA and VPA, respectively.

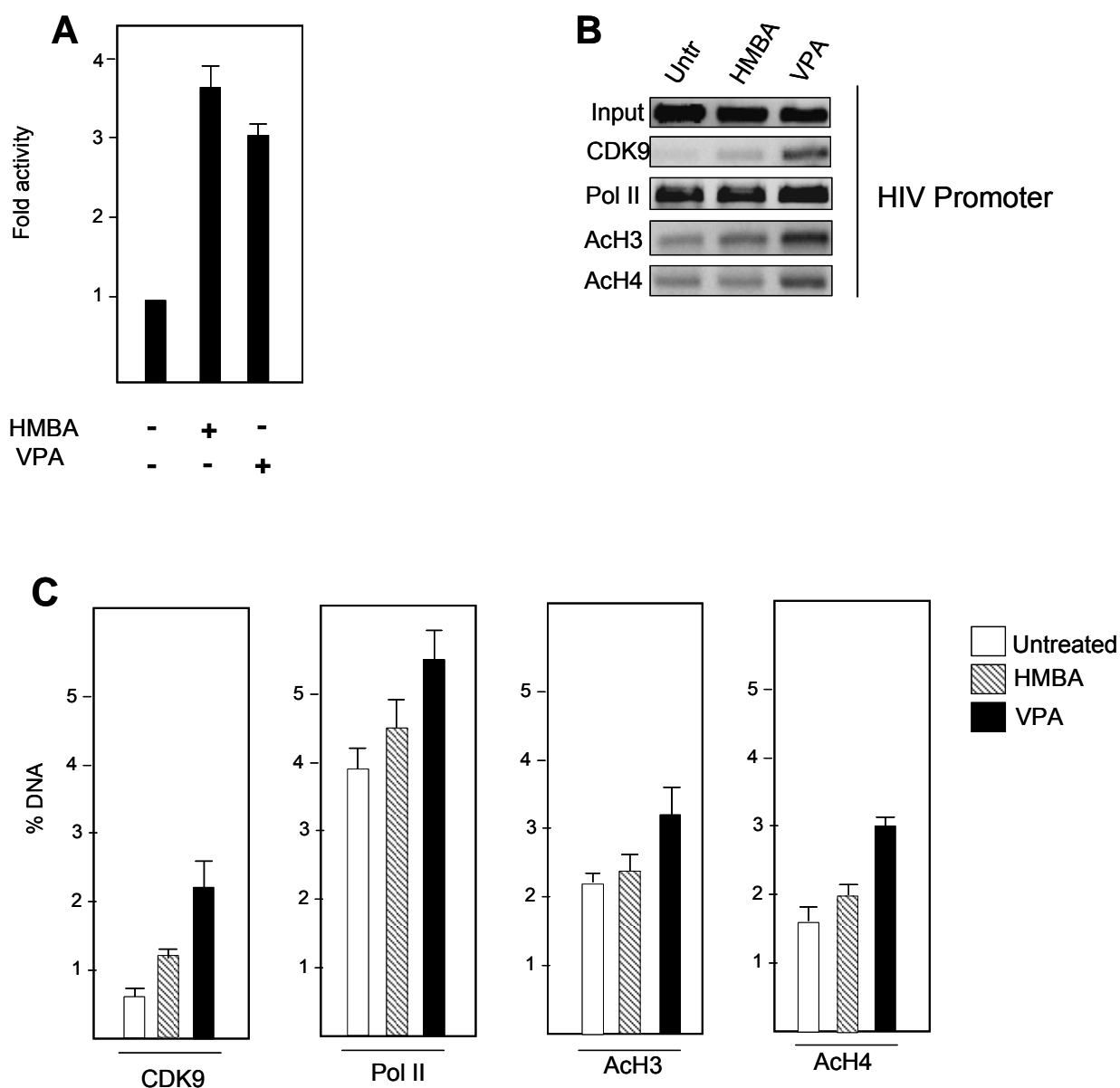


Figure 23 – Either HMBA and VPA induces Tat-independent recruitment of CDK9 to the HIV-1 promoter.

–83HIV-Luc reporter was transiently transfected into 293T cells, 24 hr after transfection cells treated with VPA and HMBA (A). After additional 24 hr cells extracts were subjected to ChIP assays using the indicated antibodies (B). The percentage of DNA immunoprecipitated relative to input is indicated with the data referring to the average of two independent experiments (C).

Aliquots of untreated, HMBA and VPA-treated 293T cells transiently transfected with HIV-1 promoter were formaldehyde crosslinked, sonicated chromatin fragments from these cells were immunoprecipitated using antibodies directed against CDK9 and RNA Polymerase II. In addition, to test possible increase in histone acetylation at viral promoter I performed ChIP using antibodies directed against the N-terminal tails of acetylated histone H3 (AcH3) and histone H4 (AcH4). As shown in figure 23 panel B and C, I found that VPA treatment leads to a clear increase of histone acetylation markedly in AcH3, whereas HMBA did not significantly modulate histone acetylation of HIV-1-LTR. Moreover, after HMBA and VPA treatments resulted in a modest increase of Polymerase II recruitment. In contrast, in the absence of treatments there was no detectable association of CDK9. However, following HMBA and VPA treatment, there was a robust increase in association of CDK9.

These findings are consistent with the possibility that either HMBA or VPA induces a Tat-independent recruitment of CDK9 to the HIV-1 promoter.

3.1.7. HMBA induces cell growth arrest and up-regulation of p21

The results reported above strongly suggest that HMBA induces expression of HIV-1-LTR driven gene expression independently from Tat. Thus, in the following section I sought to determine whether HMBA might modulate expression of cellular genes as observed for viral LTR.

Hexamethylbisacetamide (HMBA) was originally developed as an anticancer drug (Reuben and Marks, 1981), while HMBA is structurally related to HDACs inhibitors, it does not inhibit HDACs or induce histone hyperacetylation (Richon *et al.*, 1998) as shown in figure 23. Indirect evidence suggests that HMBA and HDACs inhibitors induce cell differentiation by different pathways (Richon *et al.*, 1996). It has demonstrated that HMBA induces cell cycle arrest, differentiation and/or apoptosis in various cell types (Marks *et al.*, 1987). Treatment with HMBA blocks cell cycle progression in G1 (Byers *et al.*, 2005). As shown in figure 24, treatment of huHL6 cells with HMBA induced a G1 arrest. It is known that cycling cells are continuously monitoring both internal and external conditions, and, if required, their cell cycle progression can be halted at so-called cell cycle checkpoints by arresting signals from outside or from inside the cells (Wade 2001). One of the best-known sensors and regulators of the internal cell cycle control pathway is the tumor suppressor p53 whose upregulation after DNA-damage

leads to growth arrest at least partly via induction of p21 (*El Kharroubi et al., 1998*). Consistent with this observation, I found by Western blot analysis, a concomitant increase of p21 expression, whereas levels of p53 were modestly affected (figure 25). As expected, HEXIM1 expression was induced by HMBA, whereas did not produce significant differences in the expression of the core P-TEFb subunits.

Collectively, my data strongly suggest that HMBA treatments lead to activation of HIV-1-LTR and cellular p21 gene expression. It is pertinent to note that HIV-1-LTR-driven transcription, as well as p21 gene expression, are mainly regulated at post initiation step (*Espinosa et al., 2003*); consequently I sought to determine a putative role of P-TEFb in HMBA-induced p21 expression. An approach to investigate this role, were represented by inhibition of P-TEFb complex i.e. co-expression of F:HEXIM1 and cc-CDK9 in cells expressing either HIV-1 or p21 promoters.

As shown in figure 26 panel A, HMBA treatment activates both p21 and HIV-1 promoters transiently transfected in 293T cells; however, co-expression of F:HEXIM1 effectively debilitated the activity of HMBA on both promoters constructs (figure 26 panel B). Furthermore, 293T cells where transfected with p21-Luc or the HIV-1-Luc reporters in the presence or absence of F:cc-CDK9 (that triggers the formation of large molecular weight hetero-complexes leading to a functional inactivation of the CDK9/CyclinT1 (*Napolitano et al., 2003*)) , then cells were treated with HMBA. With this approach I determined that expression of CC-CDK9 molecules effectively inhibits HMBA activity as shown in figure 26 panel C.

Despite of inherent limitations of transient transfections, which relay on the use of over-expressed proteins, these observations are indicative of the crucial role of P-TEFb in HMBA induced activation of HIV-1 and p21 gene expression.

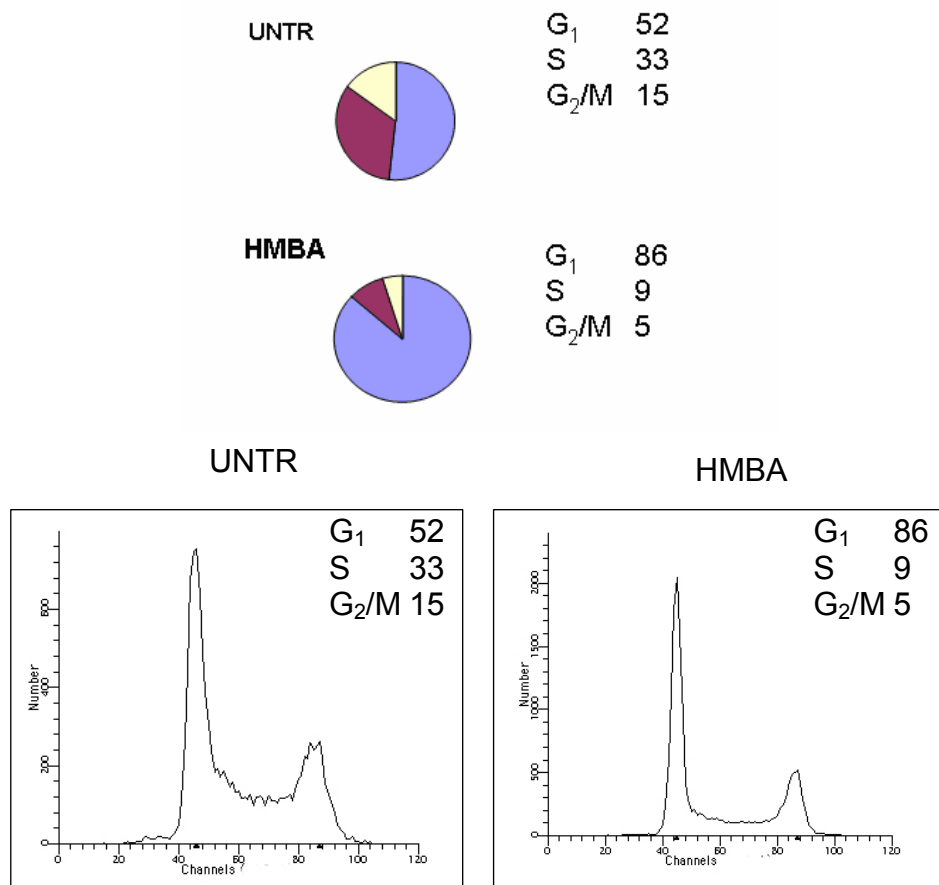
A**B**

Figure 24 – Treatment of huHL6 cells with HMBA induces a G₁ phase cell cycle arrest.

Panel A: Immunoblots were performed in 293T cells transfected with G5-83luc reporter to measure levels HEXIM1 after HMBA treatment after 24hrs. As control it performed a western blot for β -actin protein level.

Panel B: Sub-confluent 293T cells were treated for 24 hr with HMBA and VPA as indicated cells were collected and their DNA content was determined by FACS analyses. Pie charts display the percentage of cells in each stage of the cell cycle. The experiment was performed twice and resulted in similar data. Sub-confluent HL6 cells were treated for 24 hr with HMBA as indicated; cells were collected and used for FACS analyses. The experiment was performed twice and resulted in similar data.

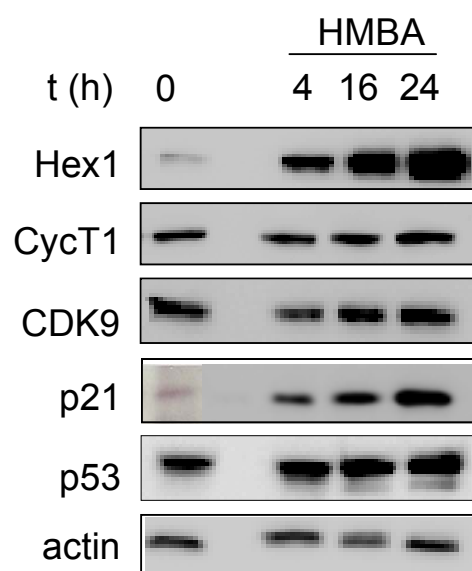


Figure 25 – HMBA induces an increase of p21 expression, whereas levels of p53 were modestly affected.

huHL6 cells extracts were prepared after HMBA treatments for indicated times (hr) and analyzed by Western blots with indicated antibodies .

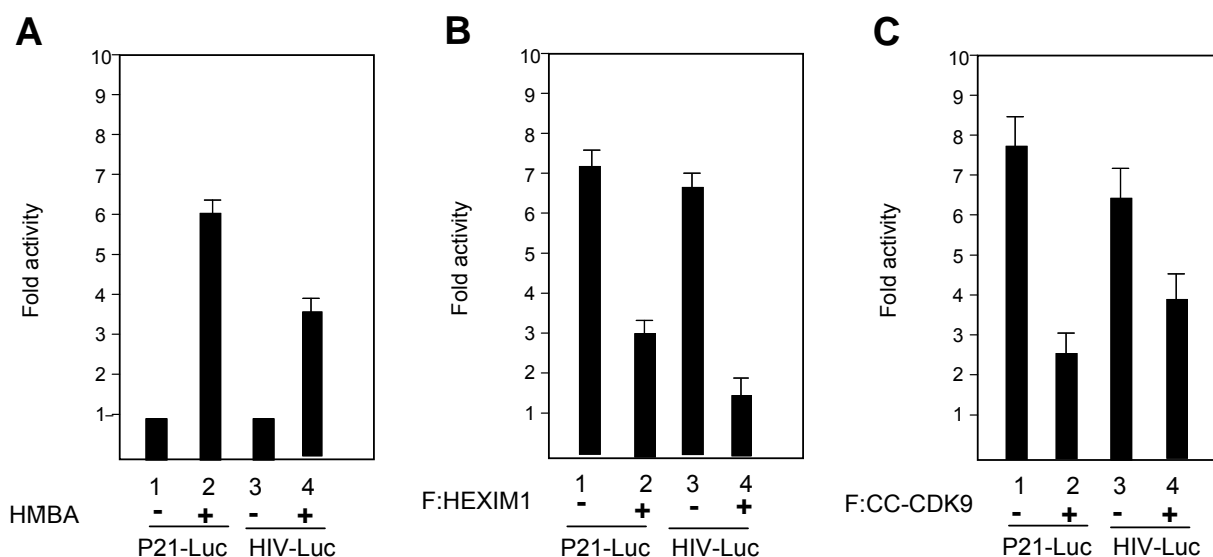


Figure 26 – The role of P-TEFb in HMBA induced activation of HIV-1 and p21 gene expression.

293T cells were transiently transfected with p21-Luc and HIV-luc reporter, respectively. Cells were then treated (24hrs after transfection) with HMBA (10mM) for additional 24hr. The level of induction in luciferase activity compared to that in untreated cells set to one is shown (panel A). In panel B and C, p21-Luc and HIV-Luc reporters were transfected into 293T cells in the presence or absence of FLAG-HEXIM1 (panel B) or FLAG-CC-CDK9 (panel C) as indicated. After 24hrs cells were treated with HMBA for additional 24hr. Cell lysate were analyzed for luciferase activities and immunoblotting with the anti-HEXIM1 (B) or anti-FLAG (C). Each histogram bar represents the mean of at least three independent transfections.

3.1.8 HMBA affects association between P-TEFb and HEXIM1

HMBA-treatment of virtually any cell line tested results in induction of HEXIM1 protein (*Price et al., 2000*). Paradoxically, HMBA treatment of huHL6 cells induces expression of HEXIM1 with a concomitant up-regulation of P-TEFb-dependent promoter activity. Because HEXIM1 together with 7SK RNA sequesters the core active P-TEFb in an inactive 7SK-HEXIM1/P-TEFb complex, I examined the association of HEXIM1 to P-TEFb by co-immunoprecipitation. Cell extracts prepared from huHL6 cells, were immunoprecipitated with anti-CDK9 and the presence of the associated CycT1 and HEXIM1 was evaluated by western blotting. HMBA treatment for 16 or 24 hours did not result in detectable differences in CDK9-associated proteins (figure 27, panel A). In contrast, cell extracts prepared after a short treatment with HMBA (1,2 and 4 hours) caused a significant reduction of HEXIM1 associated with the immunoprecipitated CDK9 (Fig.27, panel A).

Notably, the CDK9-CycT1 complex was unaffected. Thus, HMBA treatment specifically induces a rapid and transient dissociation of HEXIM1/P-TEFb complex, without altering the CDK9/CycT1 heterodimer stability.

Moreover, in collaboration with Dr.G.Napolitano, increased kinase activity was only seen in samples derived from short period of HMBA treatment (1 and 2 hrs) (data not shown and *Napolitano et al., 2007*). Altogether, the results demonstrate that HMBA leads to a rapid and transient dissociation of HEXIM1 from P-TEFb with a concomitant enhancement of CDK9 kinase activity.

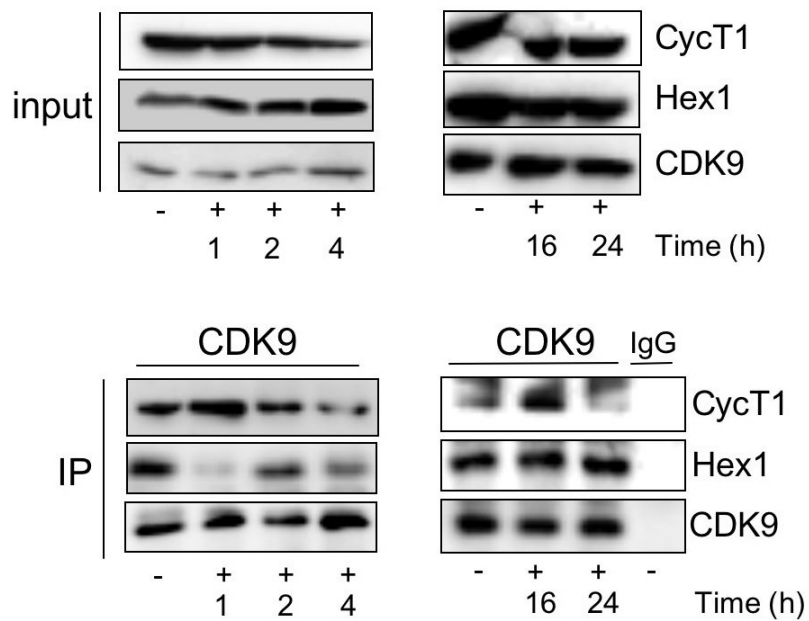


Figure 27 – HMBA leads to a dissociation of HEXIM1 from P-TEFb with a concomitant enhancement of CDK9 kinase activity.

Cell extracts prepared from huHL6 cells were immunoprecipitated with anti-CDK9 and the presence of CycT1 and HEXIM1 was evaluated by western blotting.

3.1.9 p53 interacts exclusively with “core” active CycT1/CDK9 complex

As presented above, HMBA treatment leads to activation of p21 locus and G1 arrest. Because it is well documented the activation of the p53-p21 axis in response to a variety of stimuli leading to cell cycle arrest, we investigated whether p53 might mediate the P-TEFb recruitment at p21 locus following HMBA treatment. A prerequisite for such hypothesis is the interaction between p53 and P-TEFb. To test such premise, p53^{-/-} H1299 cells were transiently transfected with CMV-p53 and CMV-CycT1 expression vectors and the cell extracts were prepared and subjected to co-immunoprecipitation (Co-IP) with anti-CycT1 or anti-p53, respectively. As reported in figure 28, panel A, in addition to the expected CDK9 and HEXIM1 proteins, p53 was found in the immunoprecipitated CycT1 materials. Reciprocally, anti-p53 antibody co-precipitated CycT1 and CDK9 proteins, however, no HEXIM1 protein was detectable in the p53-containing complex. Next, to ascertain whether endogenous p53 interacts with P-TEFb, co-IP analysis was carried out with cell extracts from p53 expressing U2OS cells. In figure 28, panel B, anti-p53 antibody co-precipitated CycT1 and CDK9, but preimmune serum did not. Reciprocally, anti-CycT1 co-precipitated endogenous p53, as well as the expected partners CDK9 and HEXIM1, whereas control IgG did not.

The absence of HEXIM1 protein in both endogenous and p53-over-expressed associated materials suggest that p53 interacts only with catalytic active P-TEFb “core” complex, a situation which is reminiscent of that observed with other P-TEFb-interacting factors such as tat and Brd4 (*Yang et al., 2005; Jang et al., 2005*).

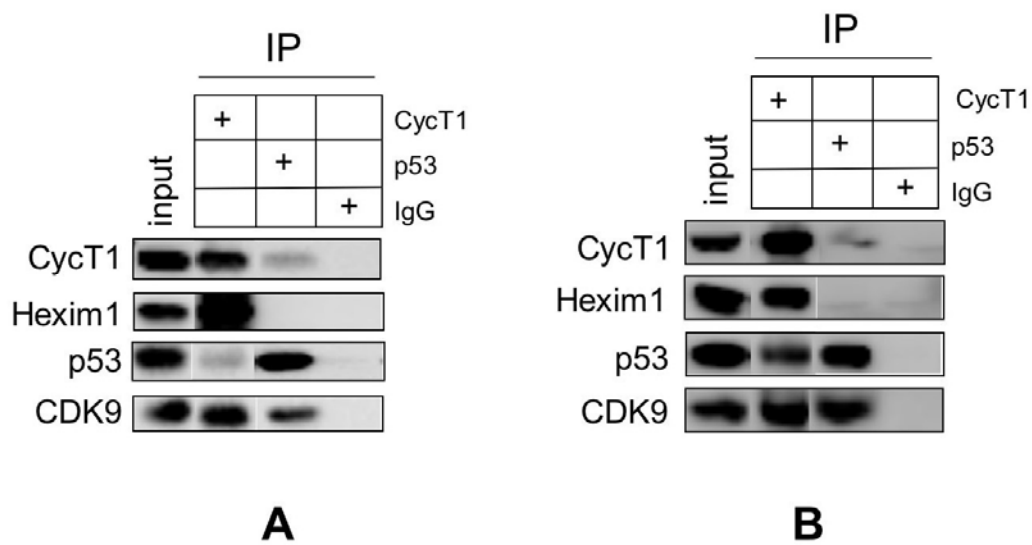


Figure 28: p53 interacts only with the catalytic active P-TEFb "core complex".

(A) Cell extracts from H1299 cells transiently transfected with p53 and CycT1 constructs were immunoprecipitated with anti p53 or anti CycT1, as indicated, and inputs and precipitates (IP) were analyzed by immunoblotting with the indicated antibodies. (B) U2OS cellular extracts were precipitated with anti p53 or anti CycT1, as indicated; inputs and precipitates (IP) were analyzed by immunoblotting with the indicated antibodies.

3.1.10 HMBA enhances recruitment of p53 and CDK9 at p21 locus

The foregoing observations suggest that HMBA-activation of p21 expression involves P-TEFb. Thus, we sought to investigate the presence of P-TEFb at the target promoter using chromatin immunoprecipitation (chIP). After treatment of huHL6 cells with HMBA for 10 hrs, chromatin was prepared and subjected to chIP assays with antibodies specific for CDK9 and p53.

Precipitated DNA was analyzed by PCR with primers spanning four p21 sequences, i.e. the affinity p53-binding site (-2283), two regions proximal to the transcription start site (-20 and +182) and the distal amplicon at + 5794. The semiquantitative nature of these assays was taken into account by performing PCR amplification using serial dilutions of DNA template as well as by repeating the experiments (3-4 times) using different chromatin preparations. Normal serum and input DNA values were used to subtract/normalize the values from chIP samples. ChIPs from treated cells reveal p53 recruitment at the major p53-binding site (-2283), while p53 recruitment increases only modestly at proximal core promoter (-20), and it was absent at the 3' portion of the gene. As compared to untreated cells, chIP experiments indicated that CDK9 levels rise significantly around the core promoter (-20 and +182 amplicons). Moreover, accordingly with previous studies, the presence of CDK9 in the distal region (+5794) is suggestive of the presence of P-TEFb throughout the active transcription unit (figure 29).

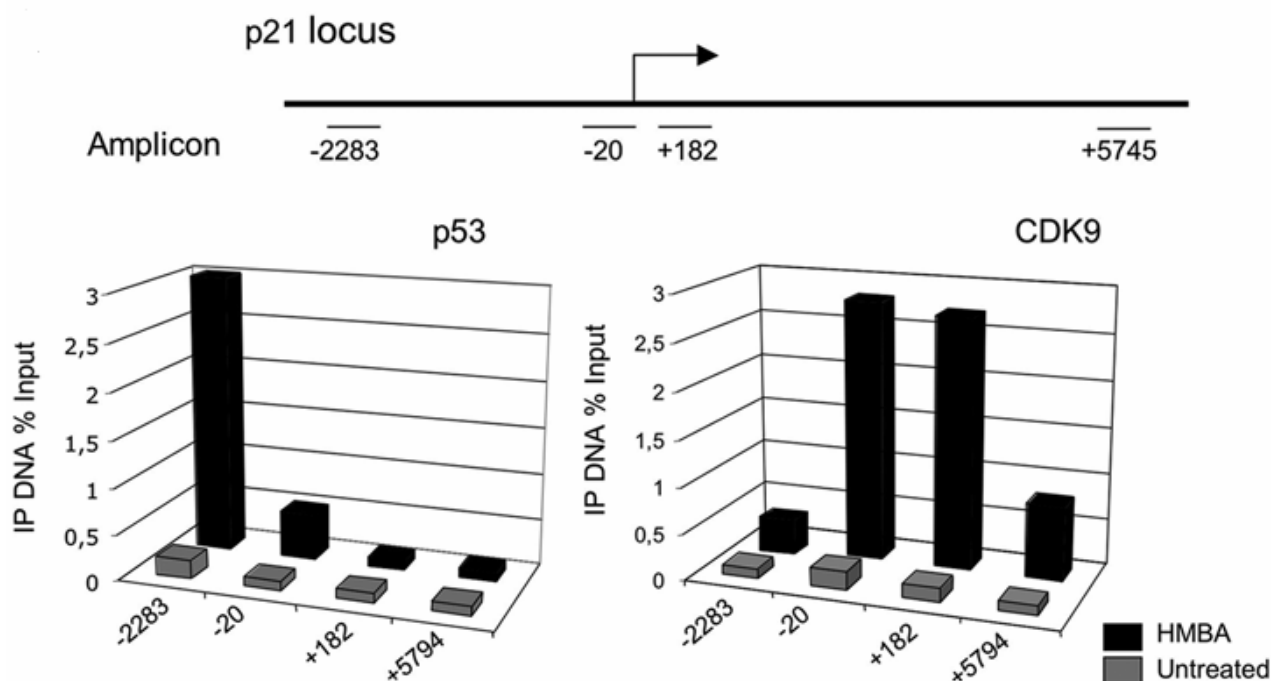


Figure 29 – HMBA enhances recruitment of p53 and CDK9 at p21 locus.

On top, schematic representation of the p21 amplicons used in the ChIP experiments. ChIP assays were performed with extracts obtained from huHL6 cells before or 10 hr after HMBA treatment with antibodies recognizing p53 and CDK9, and the relative levels of ChIP enriched DNA are shown. Values are expressed as percentage of input DNA immunoprecipitated. The results shown are the average of at least two separate immunoprecipitations from three independent cell cultures. All standard deviations were <15%.

3.2 Correlation between P-TEFb and cell cycle control

3.2.1 Relationship between P-TEFb –HEXIM1 and genotoxic insults

It has demonstrated that P-TEFb is maintained in a functional equilibrium through alternately interacting with its positive and negative regulators (*Jang et al. 2005*) although the physiological significance of this phenomenon has not been demonstrated. Nevertheless, the tight coupling of the P-TEFb equilibrium with the global control of the cell growth and differentiation agrees with the demonstrated growth-regulatory functions of the P-TEFb-associated factors, such as Myc, p53 and NF-kb (*He et al., 2006*). In the previous sections I presented evidences that HMBA induces transient dissociation of HEXIM1/7SK from P-TEFb, such circumstance is also reminiscent of the situation seen with HeLa cells treated with certain stress-inducing agents that globally disrupt transcription and suppress cell growth (*Chen et al.,2004; Michels et al.,2003; Nguyen et al., 2001;Yang et al., 2001; Yik et al., 2003*). These studies have shown that treatment of cells with the kinase inhibitor DRB causes a rapid dissociation of 7SK from P-TEFb (*Nguyen et al., 2001 and Michels et al.,2003*). Moreover, P-TEFb/HEXIM1 complex is also disrupted upon transcription arrest mediated by ActinomycinD treatment (*Michels et al.,2003*). To extend such findings I sought to look for the role of others compounds to induce P-TEFb/HEXIM1 dissociation. First, I used Doxorubicin (DXR) which induces DNA damage, but unlikely DRB, does not affect total RNAPII phosphorylation. To determine whether the DNA damage agent DXR might affect the association between HEXIM1 and P-TEFb, I performed a co-immunoprecipitation assay using a-CycT1 antibody and cell extracts were prepared from huHL6 cells treated with DRB, DXR and ActinomycinD in a dose dependent manner for two hours. The presence of HEXIM1 and CDK9 in the CycT1-IP materials was evaluated by western blotting (figure 31). I found that all treatments (DRB, DXR and ActD) affect CycT1-HEXIM1 association. In fact, less HEXIM1 was always observed in CycT1-IP from treated cells, while similar amounts of CDK9 were found in all extracts. Thus, all three agents: DRB, ActD and DXR reduce the relative amounts of HEXIM1 bound to CycT1, suggesting that these agents dissociated P-TEFb from its inhibitory subunit.

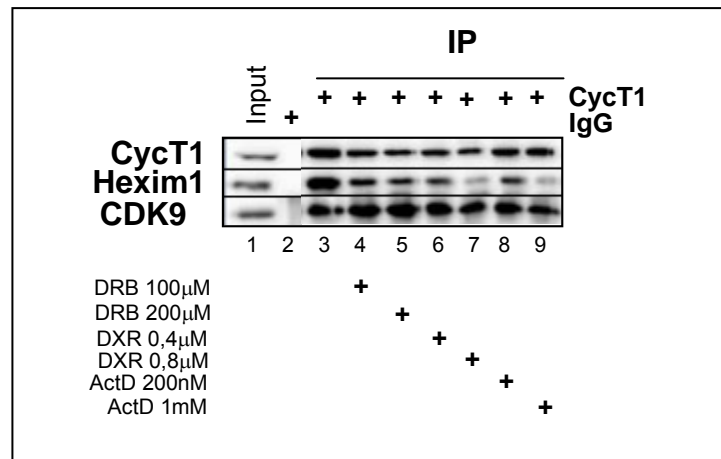


Figure 30 – DRB, Doxorubicin and Actinomycin release the active P-TEFb complex.

Cell extracts from huHL6 cells were prepared at 2 hrs after treatment with several compounds which concentrations are indicated in figure (on left). The cell extracts were immunoprecipitated with anti-CycT1, as indicated, and inputs and precipitates (IP) were analyzed by immunoblotting with the indicated antibodies.

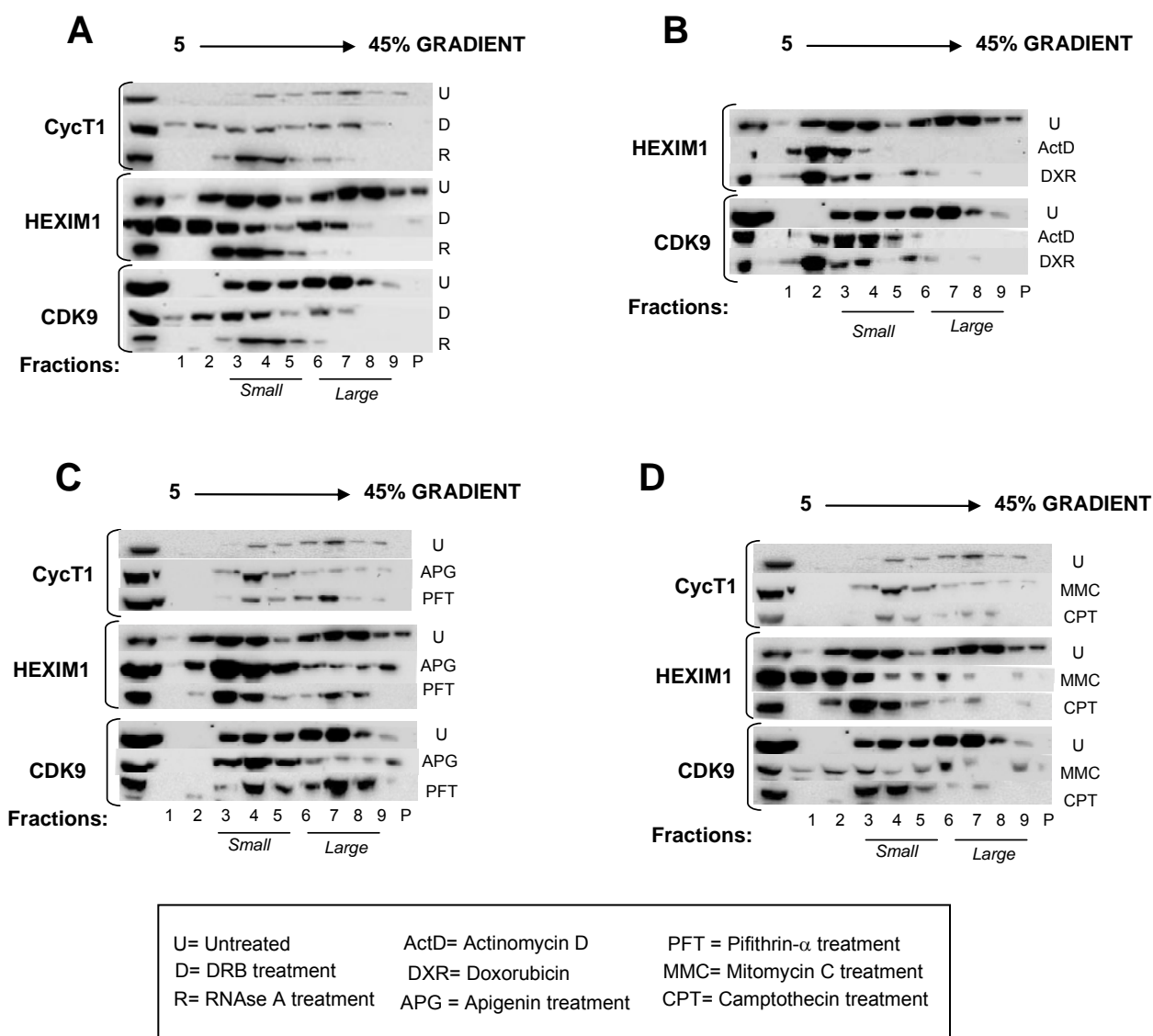


Figure 31 - Genotoxic insults shift the P-TEFb complex to inactive form and the different role of Pifithrin- α

Nuclear extracts prepared from HeLa cells treated with compounds indicated on legend, were subjected to glycerol gradient sedimentation analysis. The panels show the western detection of CycT1, HEXIM1 and Cdk9 in gradient fractions.

3.2.2 Genotoxic insults affect the P-TEFb forms in vivo

To address whether treatments with genotoxic insults could affect the ratio between small active and large-inactive P-TEFb complexes, I performed glycerol gradient assays. This assay has been commonly used to separate different P-TEFb complexes by molecular weight.

huHL6 cells were then treated with each of the following agents: DRB, DXR, ActD, Apigenin (APG), Mitomycin C (MMC), Camptothecin (CPT) and cell extracts were prepared after 2 hours of treatments and subjected to linear glycerol gradient assays. Untreated and RNase treated cells extracts, which completely released P-TEFb from HEXIM1 (*Michels et al., 2004*) were used as control.

According to previous studies, P-TEFb complexes can be detected in two major forms, the kinase active CDK9/CycT1 complex in fraction 3-4 and the large inactive CDK9/CycT1/HEXIM1/7SK complex in fraction 7-8.

As expected, inclusion of RNase disrupts the large complex (Fig.32 A, row R). Notably, I found that all these treatments, regardless their specific mode of action (see Discussion), induced dissociation of P-TEFb large complex (Fig 32, panel A, B,C,D).

Both transcriptional block induced by DRB and ActD, and genotoxic insults provoked by DXR, APG, MMC and CPT agents result in a P-TEFb dissociation. Since it is known that when DNA damage occurs, p53 is activated by ATM/ATR pathway to mediate cell cycle arrest and induce apoptosis (*Banin et al., 1998*), I can hypothesize a possible functional correlation between the ATM/ATR-p53 pathway and P-TEFb activation.

To address this point, I treated huHL6 cells with two different compounds named Pifithrin- α and Caffeine that inhibit p53 pathway affecting transcription of p53-dependent genes (p21 and Mdm2) and ATM/ATR pathway respectively.

Firstly, I performed glycerol gradient assay with extracts of huHL6 cells treated with Pifithrin- α for two hours. Pifithrin- α , is a neuroprotective drug based on p53 inhibitors (for details see Discussion), enhances cell survival after genotoxic stress such as UV irradiation and treatment with Doxorubicin, etoposide, etc, It is and a reversible inhibitor of p53 mediated apoptosis and p53-dependent gene transcription as p21 and Mdm2 (*Gudkov et al., 2005; Downer et al., 2007*). As shown in figure 32 (panel C, row PFT), no effects on P-TEFb active/inactive equilibrium were seen. Thus, I sought to look if the P-TEFb equilibrium could shift in response to the other chemical agent with similar property of Pifithrin- α , the Caffeine molecule (CAF). Caffeine is a natural stimulatory

compound that has been reported to affect cell cycle function (for details see Discussion) and to induce programmed cells death or apoptosis and perturbs key regulatory proteins as well as p53 (*He et al., 2003; Ito et al., 2003*). Most of the research literature supports that Caffeine treatments provoke an inhibition of ATM/ATR (*Zhou et al., 2000*) thus inhibiting p53 phosphorylation in response to DNA damage, resulting in p53 inactivation (*Bode and Dong 2006; Banin et al., 1998; Kastan et al., 1991*). As shown in figure 33, treatments of huHL6 cells with CAF do not affect P-TEFb equilibrium. To understand if ATM/ATR-p53 pathway is involved in P-TEFb activation in response to transcriptional block, I treated cells with Caffeine and ActinomycinD together. I sedimented cellular extracts in a linear glycerol gradient and the resulting fractions were collected from the top of the tube analyzing the results by western blot assay using antibodies direct against CDK9. As shown in figure 18, I found a P-TEFb dissociation in the presence of both Caffeine and ActinomycinD. These results strongly suggest that P-TEFb activation in response to transcriptional block is independent on ATM/ATR-p53 pathway.

In conclusion, the results here reported strongly suggest that P-TEFb is activated in response to DNA damage and transcriptional block. In fact, treatments of cells with genotoxic insults (DXR, APG, MMC and CPT) that provoke DNA damage and activation of ATM/ATR-p53 pathway, and treatments with Actinomycin D that completely block transcription, affect the ratio of P-TEFb complex versus the small-active one; furthermore, treatments with chemical compounds involved in inhibition of ATM/ATR-p53 pathway (Pifithrin- α and Caffeine), do not affect P-TEFb complex equilibrium.

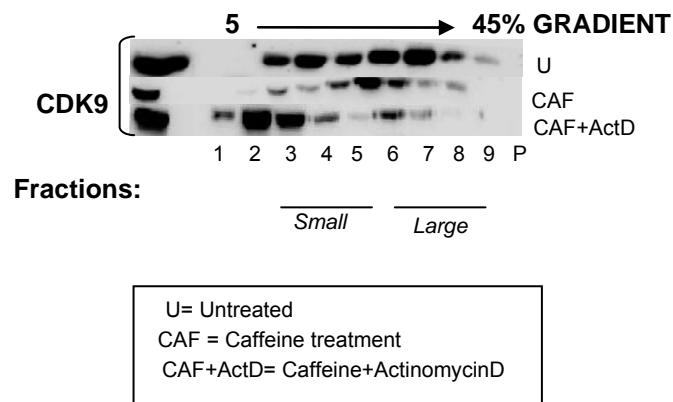


Figure 32: The effect of Caffeine of P-TEFb equilibrium after genotoxic insults.

Nuclear extracts prepared from HeLa cells treated with compounds indicated on legend, were subjected to glycerol gradient sedimentation analysis . The panels show the western detection of Cdk9 in gradient fractions.

4. Discussion

Several two past decades revealed that some of the most important mechanisms regulating eukaryotic gene expression target the movements of RNA polymerase II (RNAPII). The control of this process is predominantly mediated by an assortment of co-regulators that bridge the DNA binding factors to the transcriptional machinery, a number of chromatin-remodeling factors that mobilize nucleosomes and a variety of enzymes that catalyze the covalent modification of histones and other proteins (*Kadonaga, 2004*). Similar to the regulation of transcriptional initiation, it is likely that control of elongation occurs through the combinatorial use of elongation factors. Specifically, the elongation control of nascent transcripts seems to be have a central role in the regulation of expression of most cellular gene and the key regulatory steps controlling the entry of the RNAPII into processive elongation. This step is represented by phosphorylation state of the CTD conserved domain of RNAPII as shown in figure 33 (*Majello and Napolitano, 2001*). The CTD domain of RNAPII is the specific target for kinases that phosphorylate the RNAPII at the transition from initiation to elongation; Price and colleagues isolated a DRB positive factor P-TEFb, as an elongation factor that stimulates a shift from production of short to long transcripts (*Marshall et al., 1995*). P-TEFb is also an important RNAPII elongation factor involved in pathogenesis of HIV-1 (*Price et al., 2000*). In fact, it has been shown that P-TEFb is a significant co-factor of the HIV-1 Tat protein and that Tat activity involves direct interaction with P-TEFb complex (*Garriga and Grana, 2004*) binding to the transactivation response (TAR) element in the nascent HIV-1 transcript (*Price et al., 2000*).

Moreover, several studies demonstrated that CTD kinase activities and RNAP II phosphorylation are also differentially required for expression of distinct p53 target genes (*Gomes et al., 2006*); in fact, paused RNAPII assembles on the *p21* promoter before cellular stress and converting the elongating form upon stress-induced p53 activation (*Espinosa et al. 2003*). This process is accompanied by recruitment of several positive elongation factors and changes in CTD phosphorylation.

Many evidences, indicates P-TEFb as a complex implicated in so many different diseases (summarized in Fig. 34). It should be pointed out that various diseases may be directly or indirectly caused by dysregulation of transcription elongation which is tightly

regulated by P-TEFb/HEXIM1 (Dey *et al.* 2007). Therefore, P-TEFb complex is an important elongation factor involved either HIV-1 Tat transactivation or p53 pathway representing an attractive target for the development of novel anti-viral and in cancer therapy respectively.

In highlight of these observations, I sought to investigate the P-TEFb role in these pathway.

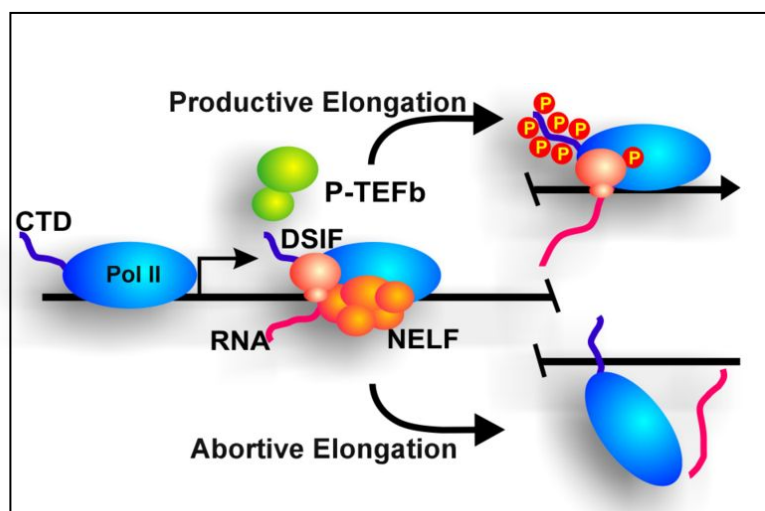


Figure 33: RNA Polymerase II Elongation Control

RNA polymerase II comes under the control of negative elongation factors (DSIF and NELF) shortly after initiation. P-TEFb mediates a transition into productive elongation by phosphorylating the CTD of the large subunit of RNA polymerase II and DSIF

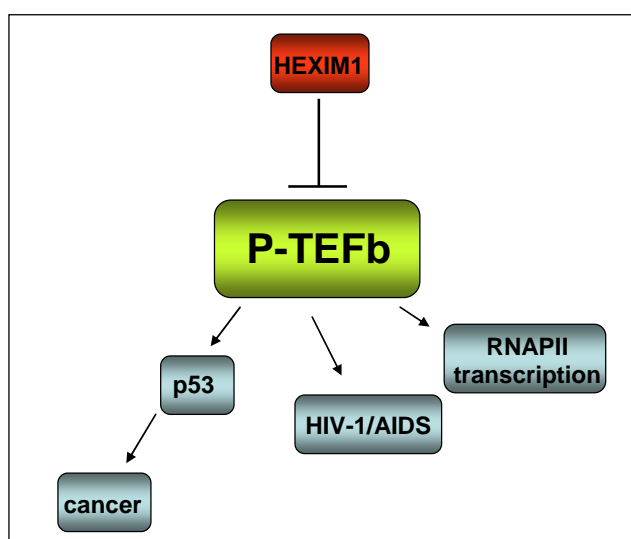


Figure 34: Role of HEXIM1 in various disease

Various disease may be directly or indirectly caused by dysregulation of transcription elongation which is tightly regulated by P-TEFb/HEXIM1

4.1 P-TEFb and HIV-1

The current model for recruitment of P-TEFb to the HIV-1-LTR predicts the formation of Tat-P-TEFb complex, which efficiently binds TAR, allowing CDK9 to phosphorylate the CTD of RNAPII, thereby enhances processivity of the RNA polymerase II to produce full-length m-RNAs (see figure 35) (*Garriga and Grana, 2004; Mancebo et al., 1997; Garber et al., 1998*). However, two different P-TEFb complexes exist in vivo in human cells (*Nguyen et al., 2001; Yang et al., 2001*): one is active and restricted to CDK9 and CyclinT1, the other is inactive and contains HEXIM1 or 2 and 7SK snRNA in addition to P-TEFb (*Michels et al., 2004; Yik et al., 2004*). It has shown that Tat interacts only with the active P-TEFb complex (*Zhang et al., 2000*), that HEXIM1 is responsive to the P-TEFb inactive complex and the presence of HEXIM1/7SK snRNA in P-TEFb complexes prevents Tat binding to P-TEFb (*Michels et al., 2003*). In highlight of these findings, I determined the functional consequence of HEXIM1 over-expression on Tat activity and the importance of 7SKsnRNA in Tat/P-TEFb inactive complex functional interaction. I demonstrated that HEXIM1 inhibits Tat-mediated transactivation of HIV-1-LTR in a dose dependent manner and that the ectopic expression of 7SK did not significantly affect HIV-1-LTR basal transcription as well as Tat-activation either alone or in a combination with HEXIM1; moreover, the specific ability of HEXIM1 to repress Tat transactivation is not affected by 7SK (figure 31). These data are consistent with a role of HEXIM1 as a negative regulator of HIV-1 activity. Moreover, I demonstrated that 7SK is not rate-limiting for the assembly of the inactive P-TEFb complex and HEXIM1-mediated inhibition of Tat activity is unlikely due to a global inhibition of P-TEFb activity.

Human HEXIM1 consists of 359 aa and is divided into four regions (as depicted in figure 21): a variable N-terminal region (1–149) that is suggested to have a self inhibitory function; a central nuclear localization signal (NLS, 150–177) that interacts with the nuclear transport machinery and directly binds to 7SK snRNA; a region of highest homology (185–220), including a negatively charged cluster that might be involved in P-TEFb inhibition and a C-terminal CyclinT binding domain (TBD) (255–359) that leads to dimerization of HEXIM1 molecules (*Yik et al., 2004; Michels et al., 2004; Shulte et al.,*

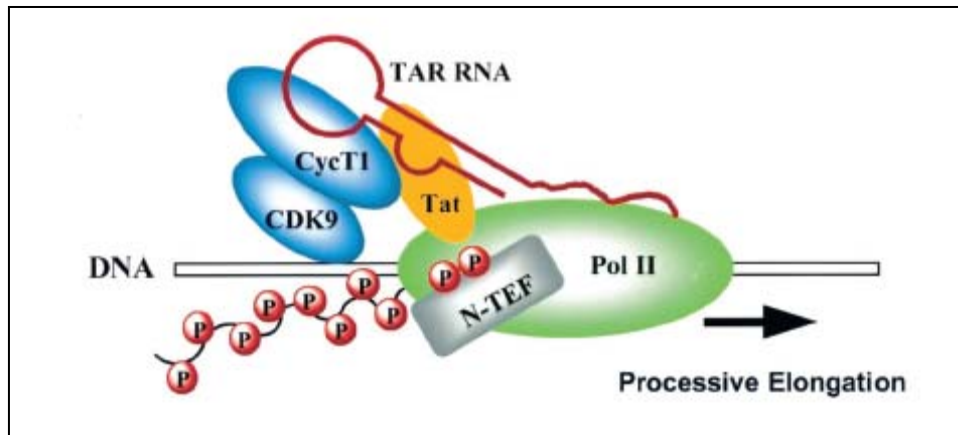


Figure 35: The model for positive transcriptional regulation on the HIV-1-LTR

Only if the viral protein Tat is present and binds to the bulge of TAR the cellular transcription elongation factor P-TEFb is recruited. Upon phosphorylation of RNAPII, NELF and DSIF by the kinase component CDK9 of P-TEFb, antitermination takes place and TAR can be elongated to the full length viral RNA.

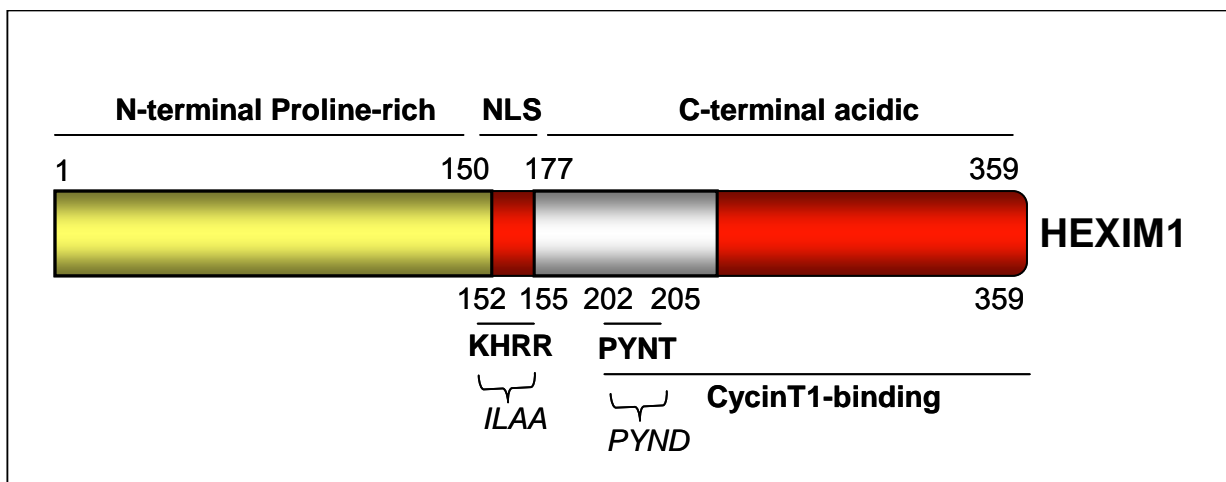


Figure 36: HEXIM1 functional domains

In figure are indicated the relevant HEXIM1 functional domains and the position of the point mutants ILAA and PYND.

2005; Barbonic *et al.*, 2005). It has been reported by Michels *et al.*, the importance of two domains of HEXIM1 protein, involved in such interactions. Specifically, the HEXIM1 C-terminal domain (181–359) is involved in the binding to P-TEFb through direct interaction with CyclinT1 by the evolutionarily conserved motif (PYNT aa202–205) and the RNA-recognition motif (KHRR) that was identified in the central region of the protein (aa 152–155), involved in a direct binding of 7SK snRNA. I delineated the important structural domains of HEXIM1 required for repression of Tat, using two HEXIM1 mutants: PYND point mutant that is impaired in repression and binding either P-TEFb or 7SK RNA in vivo, albeit it retains the ability to bind 7SK in vitro, and the HEXIM1-ILAA mutant which fails to interact in vivo and in vitro with 7SKsnRNA (Michels *et al.*, 2004). The results obtained along with previous findings strongly suggest the crucial role of 7SK in the interaction between HEXIM1 and CyclinT1. In fact, I found that, unlike wild-type HEXIM1, both mutants were unable to repress Tat albeit they were expressed at levels comparable to the wild-type protein (figure 17). Thus it appears that HEXIM1 inhibition is strictly dependent upon the integrity of the protein to interact with P-TEFb. It can be concluded that, because the point mutant ILAA abolished HEXIM1 repression on Tat, HEXIM1-mediated inhibition of Tat required the formation of the P-TEFb/HEXIM1/7SK complex. It was shown by Fraldi *et al.* that over-expression of HEXIM1 resulted in a modest reduction of P-TEFb activity (data not shown and Fraldi *et al.*, 2005), thus the inhibition of Tat activity is unlikely due to a global reduction of cellular P-TEFb activity. Later on, I demonstrated that the ectopic expression of HEXIM1 did not affect Tat expression and that exogenous expression of this results in a small but detectable reduction in Tat-bound- P-TEFb (figure 18).

Finally, because Tat and HEXIM1 interact with the cyclin-box region of CyclinT1 (figure 37), it is plausible if not likely, that the mutually exclusive interaction of these two molecules with CyclinT1 is due to binding to the same domain or to a sterical hindrance. Collectively, these data demonstrated that over-expression of HEXIM1 resulted in a modest reduction of P-TEFb activity, the inhibition of Tat activity is unlikely due to a global reduction of cellular P-TEFb activity, rather over-expression of HEXIM1 appears to influence Tat-P-TEFb interaction as depicted in figure 38.

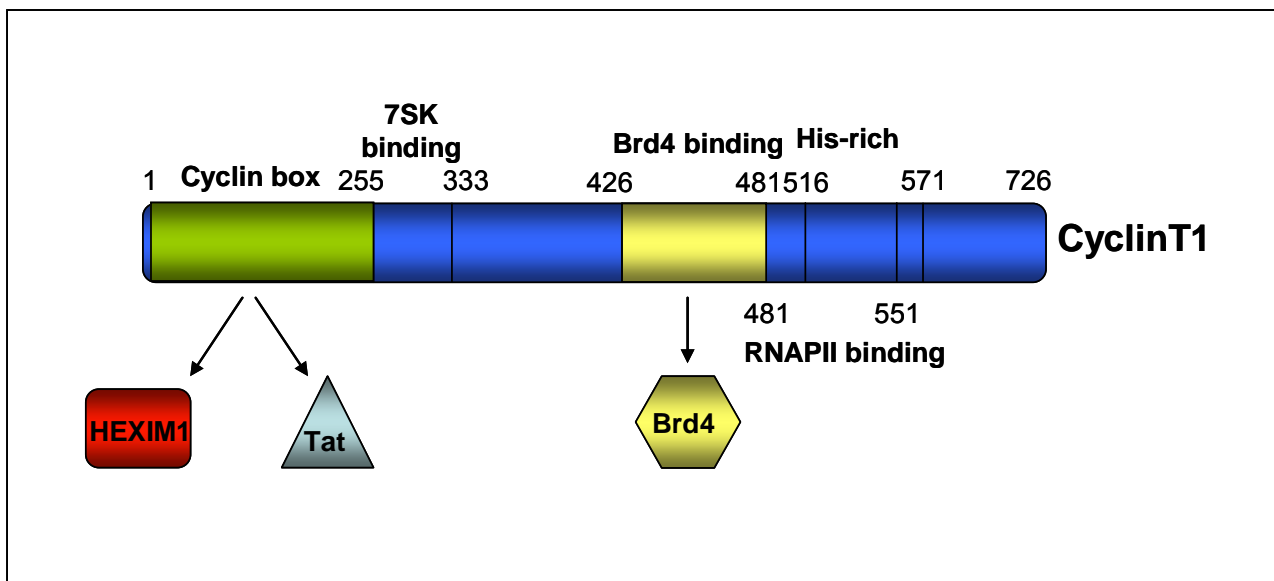


Figure 37: Diagram of human CyclinT1

CyclinT1 is a 726 amino acid protein with a cyclin box, 7SKsnRNA-interacting domain, a Brd4 binding domain and a histidine rich domain for RNAPII domain.

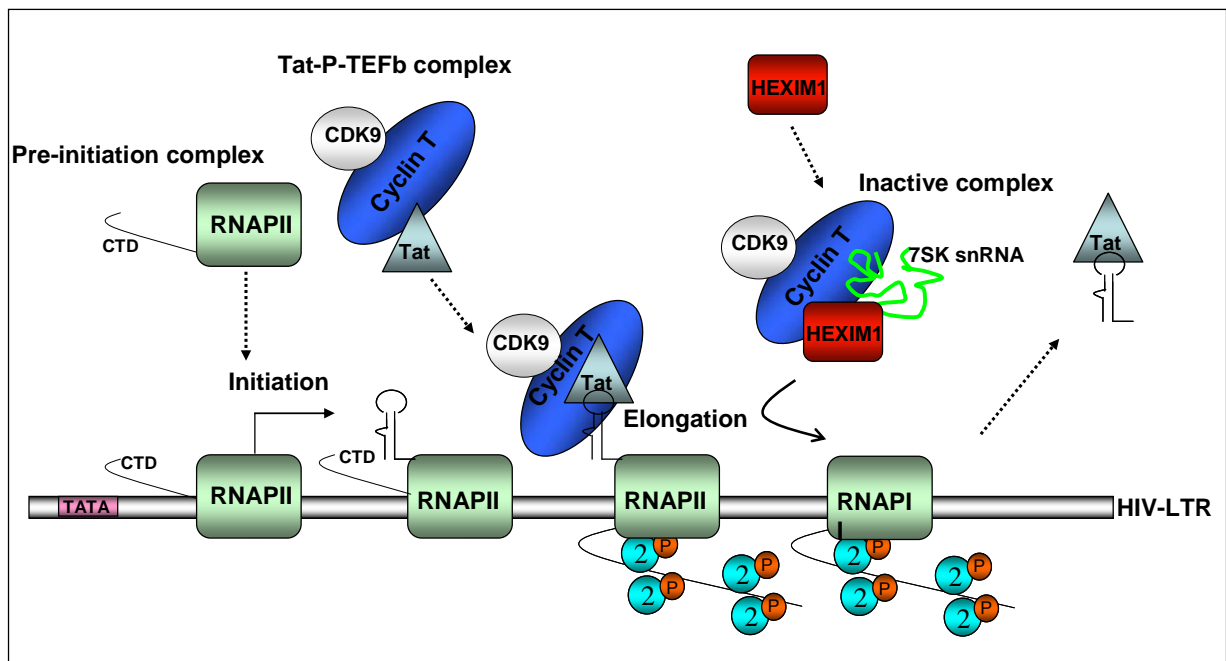


Figure 38: Schematic model for inhibition of Tat-transactivation

Over-expression of HEXIM1 results in an inhibition of Tat activity influencing Tat-P-TEFb interaction sequestering the Tat protein from P-TEFb active complex.

Recent works have demonstrated that the integrated HIV-1 provirus is assembled into an ordered chromatin structure altered by external stimuli or inhibitors of HDACs activity (*Van Lint et al., 1996; Ylisastigui et al., 2004; Williams et al., 2006*). Dynamic changes in gene expression may affect chromatin structure and, consequently, the interaction of chromatin with regulatory factors as well as HDACs inhibitors (VPA and TSA). It has demonstrated that HMBA enhances HEXIM1 expression as well as endogenous HIV-1-LTR promoter (*Zoumpourlis and Spandidos, 1992; Klichko et al., 2006*), so I investigated if HDACs inhibitors and HMBA could be enhances HIV-1-LTR basal transcription. In fact, I found that both VPA and HMBA activates basal as well Tat-mediated HIV-1-LTR expression and that both of them act like TSA to induce a robust increase of basal and Tat-activated expression of the HIV-1-LTR promoter with a concomitant amounts of HEXIM1 levels unlikely the levels of CDK9 and CyCT1 proteins that remained largely unaffected (figure 4). Recent findings, shown that HMBA-treatment of virtually any cell line tested results in induction of HEXIM1 protein (*Yik et al., 2005; Turano et al., 2006*), however, induction of HEXIM1 is not restricted to HMBA treatment but it is up-regulated by various chemical inducers, such as Retinoic acid, DMSO (*Turano et al., 2006*), VPA and TSA. Subsequently, I checked the effect of over-expression of HEXIM1 protein of the HIV-1 promoter activity in the presence of HDACs inhibitors and HMBA respectively resulting in an inhibition of the stimulatory effect exerted by these compounds, and, because both Tat and VPA stimulate basal transcription, I suggest that the inhibitory function of HEXIM1 might be due to interfering with Tat function and/or to inhibition of VPA action. In fact, I found that HEXIM1 effectively debilitates the activity of HDACs inhibitors in a Tat-independent manner, but because has an important role the identification of HEXIM1 as a P-TEFb-associated factor and P-TEFb as a principal target of HEXIM1 (*Yik et al., 2004; Michels et al., 2004*) it suggest that P-TEFb could be play a key role in HIV-1 LTR activation mediated by HDACs inhibitors in the absence of Tat. I investigated this, using another specific inhibitor of P-TEFb activity based on OCR strategy (previously described in “Results”). With this approach I demonstrated that the expression of cc-CDK9 molecules effectively inhibits VPA as well as HMBA activities (figure 21). It strongly suggests the crucial role of P-TEFb in HMBA or VPA induced activation of HIV-1-LTR promoter in the absence of Tat. These findings are in apparent contradiction with the activation of endogenous HEXIM1 protein levels observed after treatment with VPA or HMBA. However, it is pertinent to note that in human cell cultures almost half of HEXIM1 protein is not bound

to P-TEFb (*Turano et al., 2006; Yik et al., 2004; Michels et al., 2004*) , thus it is unlikely that the modest increase of endogenous HEXIM1 might affects P-TEFb activity. Several studies have highlighted the role of cis-acting DNA elements within the Long Terminal Repeat (LTR) in Tat transactivation. Although Tat stimulates mainly chain elongation, it requires cellular DNA-bound activators to stimulate transcription efficiently. In fact, mutation of cis-acting regulatory elements within HIV-1-LTR, such as Sp1 DNA-binding sites, strongly affect Tat transactivation (*Cullen et al., 1993; Jones et al., 1994; Kamine et al., 1991*). To this end, monitoring the role of cis-acting sequences in HMBA or VPA activation of HIV-1-LTR expression, I shown that transiently basal transcription of three HIV-1-LTR mutants was activated by HMBA and VPA treatments either wild-type, albeit the relative basal levels of each reporter were significantly different (figure 12). Moreover, changes in chromatin conformation were also upon treatment with HDACs inhibitors (*Wade 2001; De Ruijter et al., 2003*). The Tat mediated recruitment of HAT proteins most likely explains the changes in chromatin conformation observed at the LTR upon transactivation (*Verdin et al., 1993; Van Lint et al., 1996*). In fact, HMBA and VPA treatments activate the transcriptional activity on HIV-1-promoter carried out by the powerful approach of chromatin immunoprecipitation (chIP): I found that VPA treatment leads to a clear increase of histone acetylation markedly in AcH3, whereas HMBA did not significantly modulates histone acetylation of HIV-1-LTR, but in both these treatments I looked a modest increased RNAPII recruitment on HIV-1-LTR promoter. Notably, in contrast, in the absence of treatments there was no detectable association of CDK9 (figure 13). However, following HMBA and VPA treatment, there was a robust increase in association of CDK9. These findings are consistent with the possibility that either HMBA or VPA induces a Tat-independent recruitment of CDK9 to the HIV-1 promoter.

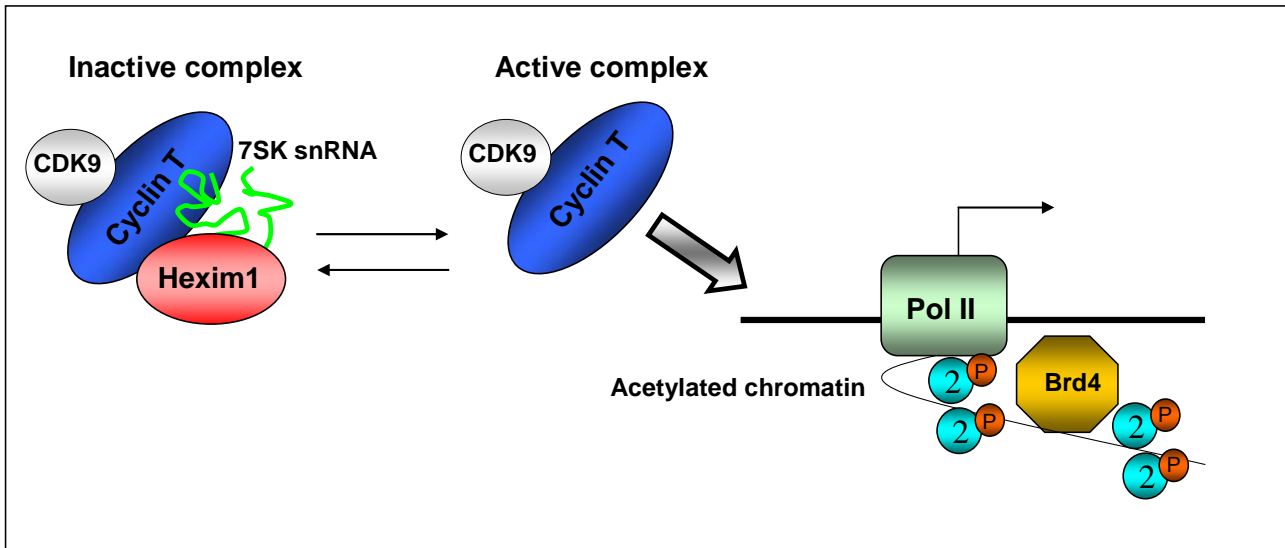


Figure 39 : a model for Brd4 recruitment of P-TEFb

P-TEFb is in a dynamic equilibrium between the Brd4 bound state and the inhibitory subunit bound state. Brd4 recruits P-TEFb to promoters in acetylated chromatin and liberates the kinase activity, allowing the transcriptional elongation.

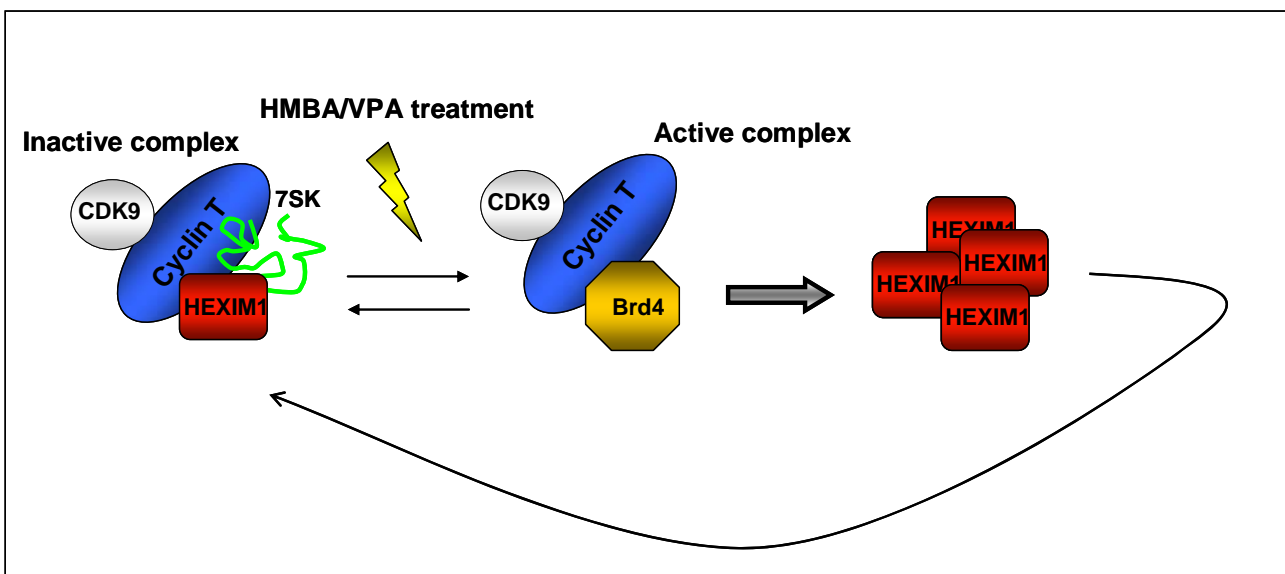


Figure 40: a model for Brd4-HEXIM1 competition to P-TEFb complex

HMBA or VPA treatments induce over-expression of HEXIM1 that may counteract the binding of Brd4 to P-TEFb, through a competitive association between the ectopic expressed HEXIM1 and P-TEFb.

Moreover, chIP assays clearly demonstrated that P-TEFb is recruited at the HIV-1 promoter after VPA treatment, in a Tat-independent manner. It remains obscure how P-TEFb is recruited at HIV-1 promoter in the absence of Tat. It has been reported that Sp1 directly interacts with CyclinT1 and that Sp1 is necessary and sufficient to recruit P-TEFb to the HIV-1LTR (*Yedavalli et al., 2003*). Moreover, P-TEFb has been shown to interact directly with the RNAPII CTD through a histidine rich region of CycT1 (*Taube et al., 2002*). It remains to be shown whether VPA treatment might enhance such interactions. An alternative mechanism for Tat-independent recruitment of P-TEFb after VPA treatment might involve interaction between P-TEFb and Brd4. Brd4 is a bromodomain protein with an highly affinity for acetylated histone H4 and H3 (*Dey et al., 2003*). Most importantly, Brd4 can simultaneous interact with both acetylated histones and P-TEFb (*Yang et al., 2005; Jang et al., 2005*). It is plausible that VPA -induced acetylation of histone tails H3 and H4 might in turn increase binding to the Brd4-P-TEFb complex leading to efficient transcription of HIV-1-LTR in the absence of Tat. Since Brd4 and HEXIM1/7SK snRNA associate in two mutually exclusive CycT1/CDK9 complexes (*Yang et al., 2005*), over-expression of HEXIM1 may counteract the binding of Brd4 to P-TEFb, through a competitive association between the ectopic expressed HEXIM1 and P-TEFb (figure 38 and 39). Finally I found that over-expression of exogenous HEXIM1, which is in association with 7SK snRNA, constitute the physiological inhibitor of P-TEFb (*Turano et al., 2006; Yik et al., 2004; Michels et al., 2004*) and inhibits HMBA, TSA and VPA-mediated activation of HIV-1 promoter. The critical role of P-TEFb in VPA-induced activation of HIV-1 promoter has been further substantiated by the ability of the negative-dominant cc-CDK9 fusion (*Napolitano et al., 2003*) to abolish HIV-1 expression in the presence of VPA. Reactivation of HIV-1 from post-integration latency by VPA has profound clinical implications, further experiments aimed at the specific study of VPA activation of HIV-1-LTR expression are necessary to elucidated this molecular mechanism.

4.2 P-TEFb and cell cycle control

While HMBA is structurally related to HDACs inhibitors, it does not inhibit HDACs or induces histone hyperacetylation (*Richon et al., 1998*). Indirect evidence suggests that HMBA and HDACs inhibitors induce cell differentiation by different pathways (*Richon et al., 1996*). It has demonstrated that HMBA induces cell cycle arrest, differentiation and/or apoptosis in various cell types (*Marks et al., 1987*). Moreover, treatment with HMBA blocks cell cycle progression in G1 (*Byers et al., 2005*). As shown in figure 9, treatment of huHL6 cells with HMBA induced a G1 arrest. It is known that cycling cells are continuously monitoring both internal and external condition, and, if required, their cell cycle progression can be halted at so-called cell cycle checkpoint by arresting signals from outside or from inside the cells (*Wade 2001*). One of the best-known sensors and regulators of the internal cell cycle control pathway is the tumor suppressor p53 whose upregulation after DNA-damage leads to growth arrest at least partly via induction of p21 (*El Kharroubi et al., 1998*). Consistent with this observation, I found a concomitant increase of p21 expression, whereas levels of p53 were modestly affected (figure 15). As expected, HEXIM1 expression was induced by HMBA, whereas did not produce significant differences in the expression of the core P-TEFb subunits.

Collectively, my data strongly suggest that HMBA treatments lead to activation of HIV-1-LTR and cellular p21 gene expression. In fact, HMBA treatment activates both p21 and HIV-1 promoters, co-expression of two P-TEFb's inhibitors, as well as HEXIM1 and cc-CDK9, effectively debilitated the activity of HMBA on both promoters constructs (figure 11). Moreover, I found that HMBA is capable to modulate CDK9 activity. In fact, it demonstrated that a CDK9 activity is largely unaffected following 24h or longer HMBA treatment (*Turano et al., 2006*). Thus, I investigated a possible change in CDK9 kinase activity after HMBA treatment for short period. I found that HMBA treatment for 16 or 24 hours did not result in detectable differences in CDK9-associated proteins. In contrast, cell extracts prepared after a short treatment with HMBA (1,2 and 4 hours) caused a significant reduction of HEXIM1 associated with the immunoprecipitated CDK9. Thus, HMBA treatment specifically induces a rapid and transient dissociation of HEXIM1/P-TEFb complex, without altering the CDK9/CycT1 heterodimer stability. Later on, I evaluated the relative amounts of associated HEXIM1 after HMBA treatments. I found that HMBA treatments caused a significant reduction of the amounts of HEXIM1

associated with the immunoprecipitated CDK9 (see figure 12), indicating that HMBA-mediated dissociation of HEXIM1 from the P-TEFb complex could be attributed to a direct interaction between HMBA and P-TEFb. Studies performed in collaboration with Dr.G.Napolitano, demonstrated an increase of kinase activity only seen in samples derived from short period of HMBA treatment (1 and 2 hrs) (data not shown and *Napolitano et al., 2007*). Altogether, the results demonstrate that HMBA leads to a rapid and transient dissociation of HEXIM1 from P-TEFb with a concomitant enhancement of CDK9 kinase activity.

As presented above, HMBA treatment leads to activation of p21 locus and G1 arrest. Because the major molecular response to HMBA exposure is the rapid increase in the amounts as well as in the catalytic activity of the P-TEFb and it is well documented the activation of the p53-p21 axis in response to a variety of stimuli leading to cell cycle arrest. I found that P-TEFb interacts with p53 (figure 13) and it makes a coherent argument on the role of p53 in mediating P-TEFb recruitment on p21 locus after HMBA treatment. The absence of HEXIM1 protein in both endogenous and p53-over-expressed associated materials suggest that p53 interacts only with catalytic active P-TEFb “core” complex, a situation which is reminiscent of that observed with other P-TEFb-interacting factors such as Tat and Brd4 (*Yang et al., 2005; Jang et al., 2005*). Thus, I presented evidences shown in figure 14, that HMBA treatment induces activation of p21 and recruitment of P-TEFb to the p21 locus, throughout the active transcription unit, underlined by the presence of CDK9 in the distal region. It is not surprising that P-TEFb is recruited to the p21 locus it has been demonstrated that p21 is regulated at post-initiation step (*Espinosa et al., 2003*). Thus, how P-TEFb is recruiting at p21 locus, is an important issue.

It has demonstrated that P-TEFb is maintained in a functional equilibrium through alternately interacting with its positive and negative regulators (*Jang et al. 2005*) although the physiological significance of this phenomenon has not been demonstrated clearly. It was found that HMBA agent is known to affect the P-TEFb equilibrium; it has been identified as the only one that can shift the P-TEFb balance toward the HEXIM1/7SK-bound state to increase the formation of the inactive P-TEFb complex (*Chen et al. 2004*). Nevertheless, the tight coupling of the P-TEFb equilibrium with the global control of cell growth and differentiation agrees well with the demonstrated growth-regulatory functions of the P-TEFb-associated factors (*He et al., 2006*).

Moreover, it has demonstrated that the HMBA induced dissociation of HEXIM1/7SK from P-TEFb is also reminiscent of the situations seen with HeLa cells treated with certain stress-inducing agents that globally disrupt transcription and suppress cell growth (*Chen et al., 2004; Michels et al., 2003; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003*). Moreover, it has been shown that treatment of HeLa cells with the kinase inhibitor DRB causes a rapid dissociation of 7SK from P-TEFb (*Nguyen et al., 2001 and Michels et al., 2003*) as well as Actinomycin D that disrupts P-TEFb/HEXIM1 by transcription arrest (*Michels et al., 2003*). Based on these observations and because HEXIM1 together with 7SK RNA sequesters the core active P-TEFb in an inactive 7SK-HEXIM1/P-TEFb complex, I found that all treatments affect on P-TEFb/HEXIM1 complex in particular, treatments of huHL6 cells with DRB provoke a reduction of P-TEFb large form within the cells in a dose independent manner while ActinomycinD provoke a reduction of P-TEFb large form within the cells lightly, in a dose dependent manner (see figure 15). The results here presented suggest that treatment of huHL6 cells with DRB and ActinomycinD acts specifically (data not shown) and that upon these conditions, the cells are trying to compensate for the loss of P-TEFb activity by releasing more active P-TEFb from the inactive and large form. In highlight of results presented above, to address whether the huHL6 cells treatments with genotoxic insults could be shift the ratio of small to large P-TEFb complex by glycerol gradient assay that is the most commonly used method to separate different protein complexes by molecular weight. As shown in figure 16, I demonstrated that upon these genotoxic insults, HEXIM1 is associated with the inactive P-TEFb complex but not with the small core P-TEFb. Consistently, when a lysate from huHL6 cells was treated with RNase A to destroy 7SK RNA and loaded onto a glycerol gradient, neither HEXIM1 nor CyclinT1 nor CDK9 remained in fractions revealing that, upon these genotoxic insults, levels of HEXIM1 were diminished greatly and that this effect were accompanied by a simultaneous reduction in the amounts of both CycT1 and CDK9 in the same fractions; this resulted in a shift of P-TEFb complex to inactive form. The results here presented suggest that treatment of huHL6 with DRB, Doxorubicin and ActinomycinD acts trying the cells to compensate for the loss of P-TEFb activity by releasing more active P-TEFb from the large form. Following severe genotoxic damage, cells undergo either rapid or delayed death. Sensitive cells undergo death within a few hours of genotoxic insult (if the damage is not so enormous as to cause immediate necrosis), whilst damage resistant cells execute death after a delay, precipitating either from the initial G2 arrest or after a number of cell divisions (*Enerpeisa*

2001). The observation that DNA-damaging agents induce levels of p53 in cells led to the definition of p53 as a checkpoint factor. While dispensable for viability, in response to genotoxic stress, p53 acts as an "emergency brake" inducing either arrest or apoptosis, protecting the genome from accumulating excess mutations. Consistent with this notion, cells lacking p53 were shown to be genetically unstable and thus more prone to tumors (*Ko and Prives, 1996*). DNA damage evokes a cellular damage response composed of activation of stress signalling and DNA checkpoint functions. These are translated to responses of replicative arrest, damage repair, and apoptosis aimed at cellular recovery from the damage. The significance of p53 in the DNA damage responses has frequently been reviewed in the context of ionizing radiation or other double strand break (DSB)-inducing agents (*Latonen 2005*). I sought to investigate whether other genotoxic agents that acts on DNA damage and subsequently affect on p53 pathway, could act on association of HEXIM1 to P-TEFb. To this end I performed gradient glycerol with anti-tumor agents as well as, Doxorubicin, Apigenin, Pifithrin- α , Camptothecin and Mitomycin C. In particular, Doxorubicin is a compound, similary to DRB, that affects on DNA damage, but it not affects total RNAPII phosphorylation, whereas DRB treatments leads to a clear shift towards the unphosphorylated form because effectively it blocks CDK9 activity (*Gomes et al., 2007*). Apigenin is an inhibitor of global kinase activity, inhibits topoisomerase I and acts arresting cell cycle in G2/M phase inducing apoptosis related to induction of p53 (*Torkin et al. 2005*). Camptothecin is capable to bind irreversibly DNA-topoisomerase I complex leading at irreversible cleavage of DNA, induces apoptosis in many tumor cells, blocks the cell cycle cells in S-phase at low dose and cause single strand breaks in DNA that are converted in DS breaks which are lethal DNA lesion (*Lansiaux et al, 2007; Ju et al, 2007*); moreover, MitomycinC inhibits DNA synthesis, nuclear division and proliferation of cancer cells, leads to a gradual formation of DSBs chromosomal DNA, a slowdown of DNA synthesis and appearance of foci and although acts primarily during the late G1 and S phase, it is not cell cylce specific (*Mladenov,2007; McKelvie 2007; Kang, 2005*). In the end, Pifithrin- α , is a neuroprotective drug based on p53 inhibitors, enhances cell survival after genotoxic stress such as UV irradiation and treatment with Doxorubicin, ectopoxide etc, and a reversible inhibitor of p53 mediated apoptosis and p53-dependent gene transcription as p21 and Mdm2 (*Downer et al 2007*). As shown in figure 17, I found a P-TEFb shift versus inactive complex with all antitumor agents mentioned, unlikely Pifithrin- α . Notably, this compound is responsive to p53 inhibition and this suggest a heavy correlation between

p53 pathway and P-TEFb active/inactive equilibrium. Agents that cause cells to override the DNA-damage checkpoint are predicted to sensitize cells to killing by genotoxic agents. One such compound, Caffeine, uncouples cell-cycle progression from the replication and repair of DNA. Caffeine therefore serves as a model compound in establishing the principle that agents that override DNA-damage checkpoints can be used to sensitize cells to the killing effects of genotoxic drugs (*Blasina et al., 1999*). Caffeine may very be the most frequently ingested neuroactive drug in the world. It has been reported that Caffeine affects cell cycle function, induces programmed cells death or apoptosis and perturbs key regulatory proteins as well as p53 (*He et al., 2003; Ito et al., 2003*). Although the effects of caffeine have been investigated, much of the research data regarding caffeine's effect on cell cycle and proliferation seem ambiguous. One important factor may be that caffeine has been used in many cell types and under a variety of conditions and concentrations. In fact, it has found that at low concentration (1mM) it induces p53 phosphorylation and p53-dependent apoptosis, whereas at 1-2mM concentration appeared induces G1 arrest and at high concentration (2-5 mM) appeared to block G1 arrest and induce apoptosis (*Bode and Dong, 2007*). Previous results demonstrated that caffeine overrides the cell cycle effects of various chemicals such as protease inhibitors, preventing apoptosis inhibits cellular DNA repair mechanisms, but notably *Mladenov et al.* has been demonstrated that caffeine is capable to delay and reverse effect of MMC. In highlighted of this, I sought to determine if this effect could affects on P-TEFb equilibrium resulting in the same effect of Pifithryn- α . I not found a P-TEFb shift versus inactive complex as shown in figure 17. Notably, because caffeine is capable to reverse the effect of DNA damage agents I found in my experiment that this property is affected on MitomycinC and ActinomycinD too. To this end, I conclude that also this compound, as well as Pifithrin- α is responsive to p53 inhibition, suggesting a heavy correlation between p53 pathway and P-TEFb active/inactive equilibrium. Therefore, the results here reported strongly suggest that P-TEFb is activated in response to DNA damage and transcriptional block. In fact, treatments of cells with genotoxic insults (DXR, APG, MMC and CPT) that provoke DNA damage and activation of ATM/ATR-p53 pathway, and treatments with ActinomycinD that completely block transcription, affect the ratio of P-TEFb complex versus the small-active one; furthermore, treatments with chemical compounds involved in inhibition of ATM/ATR-p53 pathway (Pifithrin- α and Caffeine), do not affect P-TEFb complex equilibrium.

In conclusion, as depicted in figure 26, I suggest that either transcriptional block (upon DRB and ActinomycinD treatments), UV exposure or genotoxic insults (upon DXR, MMC and CPT treatments) induce the P-TEFb complex activation. This activity is involved in the p53 pathway (Caffeine and Pifithrin- α treatments.). Indeed, the activated p53 recruits P-TEFb on the p21 promoter to induce cell cycle arrest.

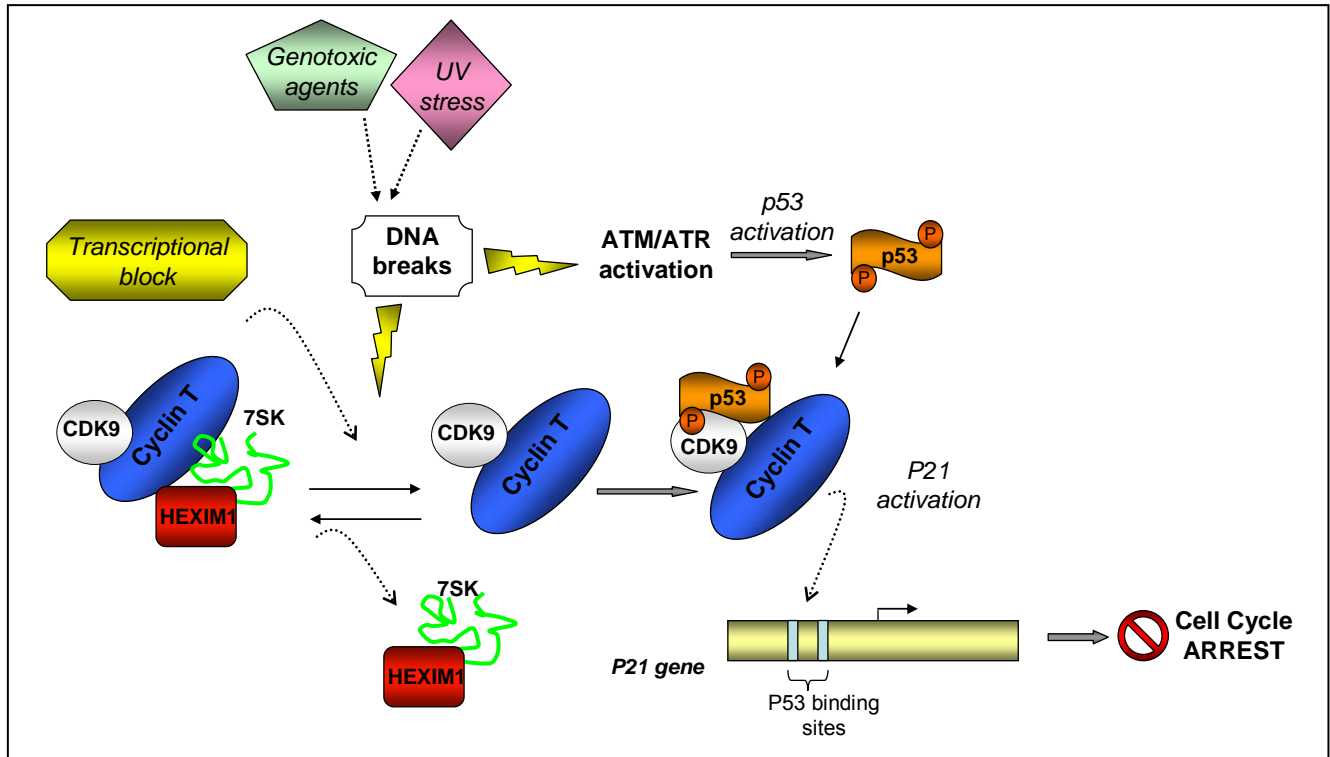


Figure 41: Schematic model of P-TEFb recruitment on p21 promoter after transcriptional block and DNA damage.

5. Material and Method

Plasmids

The G5HIV-Luc contained the HIV-LTR sequences from –83 to +82 of LTR driven the Luc gene with 5 GAL4 DNA binding sites inserted at –83 (*Majello et al., 1997*).

The pSV-Tat plasmid was described in *Majello et al. 1999*.

Gal4-CycT1 was constructed by inserting the EcoRI ± NcoI fragment from GST-CyclinT containing the full-length CyclinT1 cDNA into the SmaI site of pSG424 (*Majello et al. 1999*).

The HIV-1 long terminal repeat (LTR)-based luciferase plasmids G5-83-HIV and G5-38-HIV contain the LTR sequences from 783 to +82 and from 738 to +82 respectively with five GAL4 binding sites inserted at position 783 (G5-83-HIV) and 738 (G5-38-HIV) (*Majello et al., 1997*).

7SK snRNA plasmid was kindly provided by S.Murphy (*Murphy et al. 1992*).

The Flag tagged HEXIM1 expression vector was constructed by insertion of the corresponding cDNA regions into the EcoRV site of 3xFlag-CMV10 vector (Clontech) (*Michels et al., 2004*).

The wild-type of HEXIM1 plasmids were mutagenized by PCR to generate the deletion and point HEXIM1 mutants : ILAA, PDNT (Y203D), PYND (T205D) and PDND (Y203D, T205D) mutant proteins (*Michels et al., 2004*).

The Flag tagged cc CDK9 (3F-cc-CDK9) expression vector was constructed by insertion of PCR amplified CC domain fragment from PCDNA3-PML into the EcoRI and BglII vector (*Napolitano et al., 2003*).

The 101 flag tagged PCDNA3-Tat (PCDNA3-Tat-101-flag) expression vector was kindly provided by *Giacca*.

The p21-luciferase reporter contains the residues from 22329 to 111 of the p21 promoter (residues 2257 to 4595 of the sequence deposited in GenBank, no. U24170) subcloned from p21WWP into the HindIII site of pGL3 basic (*Pagliuca et al., 2000*).

Tissue culture and transfections

Human 293T (Human Embryonic Kidney cells), CHO (Chinese Hamster Ovary cells) and huHL6 (a clonal cell line bearing an integrated HIV-LTR-Luc reporter derived from Hela cells) (Casse *et al.*, 1999) cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco Life Technologies). 293T cells were used for transient transfection with HIV1-LTR-Luc reporter (100ng) in the presence of pSV-tat (50 ng) along with increasing (10, 100 and 500 ng) amounts of F:HEXIM1 and 7SK RNA (10, 100 and 500 ng). CHO cells were transfected with HIV-LTR-luc reporter (50ng) in the presence of pSV-Tat (100ng) and together with CMV-hCycT1 (200ng), in the presence of increasing amounts of F:HEXIM1 and 7SK.

Moreover, 50ng of G5-HIVLuc reporter (Majello *et al.*, 1994) was transfected into 293T cells along with Gal4-CycT1 (200 ng) or pSV-Tat (50 ng) along with increasing amounts of Flag:HEXIM1 wild type and mutants (10, 100 and 500 ng). After further 24 hours, cells were harvested and lysed for luciferase assays.

50ng of pCDNA3-Tat-101-flag plasmid were used for transient transfections of huHL6 cells. 24 hours after transfection the culture medium was replaced with fresh one containing or not HMBA (10mM) and VPA (5mM). After further 24 hours, cells were harvested and lysed for luciferase assays. HuHL6 cells harboring an integrated HIV-LTR-Luc reporter were transfected with pcDNA3-Tat vector (50 ng) and 24 hours after transfection the culture medium was replaced with fresh one containing or not HMBA (10mM) and VPA (5mM). Cell lysates then prepared were analyzed for luciferase activities.

Human 293T cells transfected with –83HIV-Luc reporter in the presence or absence of co-transfected pCDNA3-Tat-101flag vector (50 ng). As huHL6 cells, 24 hours after transfection the culture medium was replaced with fresh one containing or not HMBA (10mM), VPA (5mM) and TSA (100nM). Cell lysates then prepared were analyzed for luciferase activities.

293T cells were co-transfected with –83HIV-Luc reporter together with pcDNA3-Tat vector (50ng), in the presence or absence of 1µg of F:HEXIM1 vector (Fraldi *et al.*, 2005) or 1µg of F:CC-CDK9 (Napolitano *et al.*, 2003). After 24 hr cells were treated with VPA, TSA and HMBA for additional 24 hr as indicated. Cell lysates were then prepared and analyzed for luciferase activities.

For all transfection assays 2.5×10^5 cells were transfected by a liposome method (Lipofectamine reagent; Life technologies, Inc.) in 2cm/dish in multiwells.

Whole cell extract

Cells were harvested and washed in ice-cold PBS. After centrifugation, the cellular pellet were resuspended in BfrA (10mM HEPES pH 7,9, 1.5 mM MgCl₂, 10mM KCl, 200 mM NaCl, 0.2 mM EDTA) and vortexed for 30 minutes on ice. The lysates were then centrifugated at 14000rpm for 30minutes at 4°C.

Luciferase assay

Luciferase activity was quantified using Dual-Luciferase Reporter assay (Promega) according to manufacture's instructions and activity normalized in respect of the amount of protein cell extract.

FACS analysis

huHL6 cells were treated for 24hours for HMBA (10 mM). After was trypsinised and collected by centrifugation and washed in phosphated-buffered saline (PBS). Cells were resuspended in hypotonic solution (0.1% na-Citrate, 50µg/ml Propidium iodide, 6.25 µg/ml Rnase and 0.00125% Nonident P40 (Sigma Chemical Co), incubated in absence of light for 30-60 minutes at room temperature. Cell cycle data acquisition and analyses were performed on Becton Dickinson flow cytometer using Cell Quest Pro and ModFit 3.0 software.

Western blotting and antibodies

Cells were lysed in ice-chilled Buffer A (10mM HEPES pH 7,9, 1.5 mM MgCl₂, 10mM KCl, 200 mM NaCl, 0.2 mM EDTA), supplemented with 1mM dithiotreitol (DTT), 40U/ml of Rnasin (Promega), protease inhibitor cocktail (P-8340; Sigma), and 0.5% Nonident P-40. Lysates were vortexed and incubated for 20 min on ice and clarifies by centrifugations. Western blotting were performed using the following antibodies: the rabbit polyclonal anti-HEXIM1 (C4) has been previously described (*Michels et al., 2003*); goat polyclonal anti-CycT1 (T-18), rabbit polyclonal anti CDK9 (H -169) and goat polyclonal anti-Actin (I-19) were from Santa Cruz. Anti-flag M2 monoclonal antibody were from Sigma. Mouse monoclonal anti-p53 (DO-1) were from Santa Cruz and Rabbit polyclonal anti-p21 (N-20) were from Santa Cruz. Anti-Tat were from NIH AIDS Research Reagent Program. Binding was visualized by enhanced chemiluminescence (ECL-plus Kit, Amersham Biosciences).

ChIP assay

For chip assay 10 μ g of –83HIV-Luc reporter DNA plasmid was transiently transfected into 293T cells, 24 hr after transfection cells treated with VPA (5mM) and HMBA (10mM). After further 24 hours chIP assays were carried out as described (*Raha et al., 2005*). Briefly 24 hours after transfection, cell culture medium was replaced with fresh medium containing or not HMBA (10mM) and VPA (5mM). After 24hrs cells were treated as described in *Raha et al., 2005*. Cells were cross-linked by adding formaldehyde to the medium (1% final concentration) and incubated for 10 minutes at room temperature. Cells were then washed with ice-cold PBS containing protease inhibitors. Cells were then lysed in SDS-lysis buffer (1% SDS, 50mM Tris-Hcl pH8, 10mM EDTA and protease inhibitors), and sonicated at least 6-8 times for 10 sec. with 2 minutes incubation on ice between. Lysates were centrifuged and 5% of the lysates were kept as input. Lysates were diluted 10-fold with chIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7 mM Tris-Hcl pH8, 16.7 mM NaCl and protease inhibitors) and pre-cleared with 80 μ l of Salmon Sperm DNA/Protein G Sepharose 4 Fast-Flow (Amersham) for 4hrs at 4°C on a wheel and the supernatant was incubated overnight with the primary antibody : 10 μ g of goat polyclonal anti CDK9 L-19, anti-8WG16 (Babco, Richmond, CA) anti-Ach3 e Ach4 (Upstate Biotechnology), anti-p53 clone DO1 (Santa Cruz, Inc). After the overnight incubation with antibodies, 20 μ l of Salmon Sperm DNA/Protein G Sepharose were added, and the mixture was incubated for 1 hour at 4°C on a wheel. After extensive washes with low-salt immune complex wash buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mMTris HCl pH8 and 150mM NaCl) and one with high-salt immune complex wash buffer (0,1%SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris Hcl pH8 and 500mM NaCl), immuno-complexes were washed twice with TE buffer (10mM tris Hcl pH8 and 1mM EDTA). Chromatin was eluted with freshly prepared elution buffer (1%SDS and 0.1M CHNaO₃). After reversal of the cross-links (at 65°C overnight), the samples were treated with proteinaseK for 2h at 55°C, extracted by phenol/chloroform and ethanol precipitated. The pellet was resuspended in TE buffer and PCR was permormed. IP DNA was quantizated with a Gel Doc instrument (Bio-Rad Laboratories) and presented as the ratio of IP to input.

Co-immunoprecipitation assay

CoIP were carried out as previously described (*Michels et al., 2004, Turano et al., 2006*). Whole-cell extracts (1mg) were prepared from 293T cells co-transfected with pSV-Tat and F:HEXIM1. Extracts were obtained using buffer A (10mM HEPES pH 7,9, 1.5 mM MgCl₂, 10mM KCl, 200 mM NaCl, 0.2 mM EDTA)

and were immunoprecipitated with anti-CycT1 (H-245 from Santa Cruz) and normal IgG pre-adsorbed with proteinA/Sepharose beads, respectively. After extensive washes, the recovered materials were resolved on SDS-PAGE and immunoblotted with the anti-Tat (NIH AIDS Research Reagent Program), the rabbit polyclonal anti-HEXIM1 (C4) has been previously described (*Michels et al., 2003*); goat polyclonal anti-CycT1 (T-18), Anti-flag M2 monoclonal antibody were from Sigma. To detect endogenous interaction between p53 and P-TEFb cell extracts from 293T cells (1,5mg) were prepared and processed as above.

Glycerol Gradient

huHL6 cells were grown in DMEM to a density of 4×10^5 cells/ml in 10cm plates. The cells were treated for 2 hours with P-TEFb inhibitors (DRB100 μ M, RNase 10 μ and ActD 1mM) and anti-tumor agents (DXR 0,8 μ M, CPT 12 μ M, APG 60 μ M, MMC 10 μ /ml, CAF 2mM, PFT 100nM). Cell lysates were prepared in Buffer A (10 mM KCl, 10 mM MgCl₂, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1% PMSF and EDTA-free complete protease inhibitor cocktail (Roche) + RNasin 1 μ /ml Promega) containing 150 mM NaCl and 0.5% NP-40. The lysates were clarified by centrifugation at 14000 rpm for 30 minutes at 4°C. The supernatant was layered on top of a 5–45% glycerol gradient containing 150 mM NaCl. Gradients were spun at 39000 rpm for 16 hours using a SW-4Ti rotor. The fractions were analyzed for the presence of PTEFb complexes by immunoblotting with anti-CyclinT1, anti-HEXIM1 and anti-CDK9 antibodies (Santa Cruz) described above. Following incubation with the appropriate HRP-conjugated secondary antibodies, the blots were developed using enhanced chemiluminescence (ECL-plus Kit, Amersham Biosciences). The amount of P-TEFb in the large and free form was quantitated using a Gel Doc instrument (Bio-Rad Laboratories).

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7. APPENDIX

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Research

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Inhibition of Tat activity by the HEXIM1 protein

Alessandro Fraldi^{1,3}, Francesca Varrone¹, Giuliana Napolitano¹, Annemieke A Michels², Barbara Majello¹, Olivier Bensaude² and Luigi Lania*¹

Address: ¹Department of Structural and Functional Biology, University of Naples 'Federico II', Naples, Italy, ²UMR 8541 CNRS, Ecole Normale Supérieure, Laboratoire de Régulation de l'Expression Génétique, Paris, France and ³Telethon Institute of Genetics and Medicine (TIGEM) Naples, Italy

Email: Alessandro Fraldi - fraldi@tigem.it; Francesca Varrone - f.varrone@unina.it; Giuliana Napolitano - giuliana.napolitano@unina.it; Annemieke A Michels - michels@biologie.ens.fr; Barbara Majello - barbara.majello@unina.it; Olivier Bensaude - bensaude@biologie.ens.fr; Luigi Lania* - lania@unina.it

* Corresponding author

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Abstract

Background: The positive transcription elongation factor b (P-TEFb) composed by CDK9/CyclinT1 subunits is a dedicated co-factor of HIV transcriptional transactivator Tat protein. Transcription driven by the long terminal repeat (LTR) of HIV involves formation of a quaternary complex between P-TEFb, Tat and the TAR element. This recruitment is necessary to enhance the processivity of RNA Pol II from the HIV-1 5' LTR promoter. The activity of P-TEFb is regulated *in vivo* and *in vitro* by the HEXIM1/7SK snRNA ribonucleic-protein complex.

Results: Here we report that Tat transactivation is effectively inhibited by co-expression of HEXIM1 or its paralog HEXIM2. HEXIM1 expression specifically represses transcription mediated by the direct activation of P-TEFb through artificial recruitment of GAL4-CycT1. Using appropriate HEXIM1 mutants we determined that effective Tat-inhibition entails the 7SK snRNA basic recognition motif as well as the C-terminus region required for interaction with cyclin T1. Enhanced expression of HEXIM1 protein modestly affects P-TEFb activity, suggesting that HEXIM1-mediated repression of Tat activity is not due to a global inhibition of cellular transcription.

Conclusion: These results point to a pivotal role of P-TEFb for Tat's optimal transcription activity and suggest that cellular proteins that regulate P-TEFb activity might exert profound effects on Tat function *in vivo*.

Background

The positive transcription elongation factor b (P-TEFb) composed by CDK9/CyclinT1, has emerged as a significant co-factor of the HIV Tat protein. P-TEFb complex has been shown to associate with and phosphorylate the carboxyl-terminal domain (CTD) of RNA pol II, thereby enhancing elongation of transcription [1-3]. Tat protein

binds an uracil containing bulge within the stem-loop secondary structure of the Tat-activated region (TAR-RNA) in HIV-1 transcripts [4-6]. Tat functions as an elongation factor and stabilizes the synthesis of full-length viral mRNAs by preventing premature termination by the TAR-RNA stem-loop. Physical and functional interactions between Tat and P-TEFb have been well documented [7,8]. Tat

binds to P-TEFb by direct interaction with the human cyclinT1, and the critical residues required for interaction have been delineated [9,10]. The current model for recruitment of P-TEFb to the LTR, predicts the formation of the Tat-P-TEFb complex, which efficiently binds TAR, allowing CDK9 to phosphorylate the CTD of RNAPII, thereby, enhances processivity of the polymerase to produce full-length mRNAs [3,7-10].

Like other CDKs, the P-TEFb activity is regulated by a dedicated inhibitor. Two different P-TEFb complexes exist in vivo [11,12]. The active complex is composed of two subunits, the CDK9 and its regulatory partners cyclinT1 or T2. In addition, a larger inactive complex has been identified, which comprises of four subunits, CDK9, cyclinT1 or T2, the abundant small nuclear RNA 7SK and the HEXIM1 protein [13-17]. It has been recently shown that HEXIM1 has the inherent ability to associate with cyclin T1 and binding of 7SK snRNA turns the HEXIM1 into a P-TEFb inhibitor [15-17]. The relative presence of core and inactive P-TEFb complexes changes rapidly in vivo [11,12]. Several stress-inducing agents trigger dissociation of the inactive P-TEFb complex and subsequent accumulation of kinase active P-TEFb [11]. Thus, the 7SK-HEXIM1 ribonucleic complex represents a new type of CDK inhibitor that contributes to regulation of gene transcription. A further level of complexity of this system comes from the recent identification of HEXIM2, a HEXIM1 paralog, which regulates P-TEFb similarly as HEXIM1 through association with 7SK RNA [18,19].

It has been showed that Tat binds exclusively to the active P-TEFb complex [13]. Thus the presence of HEXIM1/7SK snRNA in P-TEFb complexes prevents Tat binding. Since the association between 7SK RNA/HEXIM1 and P-TEFb appears to compete with binding of Tat to cyclinT1, we have speculated that the TAR RNA/Tat system may compete with the cellular 7SK snRNA/HEXIM1 system in the recruitment of the active P-TEFb complex [13]. Accordingly, it has been shown that over-expression of HEXIM1 represses Tat function [14,17].

We show here that HEXIM1, or its paralog HEXIM2, inhibits Tat trans-activation of HIV-LTR driven gene expression, and more importantly, we demonstrated the role of the 7SK snRNA recognition motif as well as the binding to cyclin T1 as crucial elements for efficient Tat inhibition.

Results

Tat activity is inhibited by HEXIM1

Tat activity involves direct interaction with CDK9/CyclinT1 (P-TEFb) complex. However, two major P-TEFb-containing complexes exists in human cells [11,12]. One is active and restricted to CDK9 and cyclin T, the other is

inactive and it contains HEXIM1 or 2 and 7SK snRNA in addition to P-TEFb [15,17]. We have previously shown that Tat interacts only with the active P-TEFb complex [13]. Because the two complexes are in rapid exchange, we sought to determine the functional consequences of the over-expression of HEXIM1 and 7SK snRNA on HIV-1 LTR-driven gene transcription. To this end we performed transient transfections in human 293 cells using the HIV-LTR-Luc reporter in the presence of increasing amounts of Flag-tagged HEXIM1 and 7SK snRNA, respectively. Dose-dependent expression of F:HEXIM1 was monitored by immunoblotting with anti-HEXIM1 antibody (Fig. 1 panel A). As presented in Fig. 1B, we found that basal transcription from the LTR sequences was unaffected by the presence of F:HEXIM1 or 7SK RNA. In contrast, Tat-mediated transactivation of the HIV-1 LTR was inhibited by the over-expression of F:HEXIM1 in a dose-dependent manner. Ectopic expression of 7SK RNA did not significantly affected HIV-LTR-Luc expression either alone or in combination with F:HEXIM1. Thus, it appears that HEXIM1 is able to repress Tat-mediated activation. To further substantiate the inhibitory function of HEXIM1 we sought to extend our analysis using the murine CHO cells. Tat protein is a potent activator of HIV-1 LTR transcription in primate cells but only poorly functional in rodent cells [6,7]. However, Tat-mediated activation can be rescued by enforced expression of human cyclin T1 [6,7]. As presented in Fig. 1C we found that, while hCycT1 rescued Tat function, ectopic expression of HEXIM1 effectively inhibits Tat activity. Most importantly, Tat enhancement mediated by hCycT1 was effectively abrogated by co-expression of HEXIM1 in a dose-dependent manner. Finally, like in human cells, ectopic expression of 7SK snRNA did not have any significant effect on Tat activity.

The results reported above suggested that ectopic expression of HEXIM1 inhibits Tat activity. A large number of evidences indicate that Tat-transactivation is mainly due to the recruitment of the cellular complex P-TEFb to the LTR, causing phosphorylation of the RNAPII CTD [1,6-10]. Accordingly, we and others have previously showed that artificial recruitment of P-TEFb to the HIV-1 promoter is sufficient to activate the HIV-1 promoter in the absence of Tat [20,21]. We sought to determine the consequences of ectopically expressed F:HEXIM1 on P-TEFb induced transcription in the absence of Tat. We showed that direct recruitment of CyclinT1 to a promoter template by fusion to the GAL4 DNA binding domain, activates transcription from an HIV-1 LTR (G5HIV-Luc) reporter bearing GAL4 sites [20]. Human 293 cells were transfected with the G5HIV-Luc reporter along with GAL4-fusion expression vectors in the presence of F:HEXIM1. As shown in Fig. 2A, we found that GAL4-CycT1 effectively activates transcription from the HIV-1 LTR reporter, and co-expression of F:HEXIM1 resulted in a robust dose-dependent

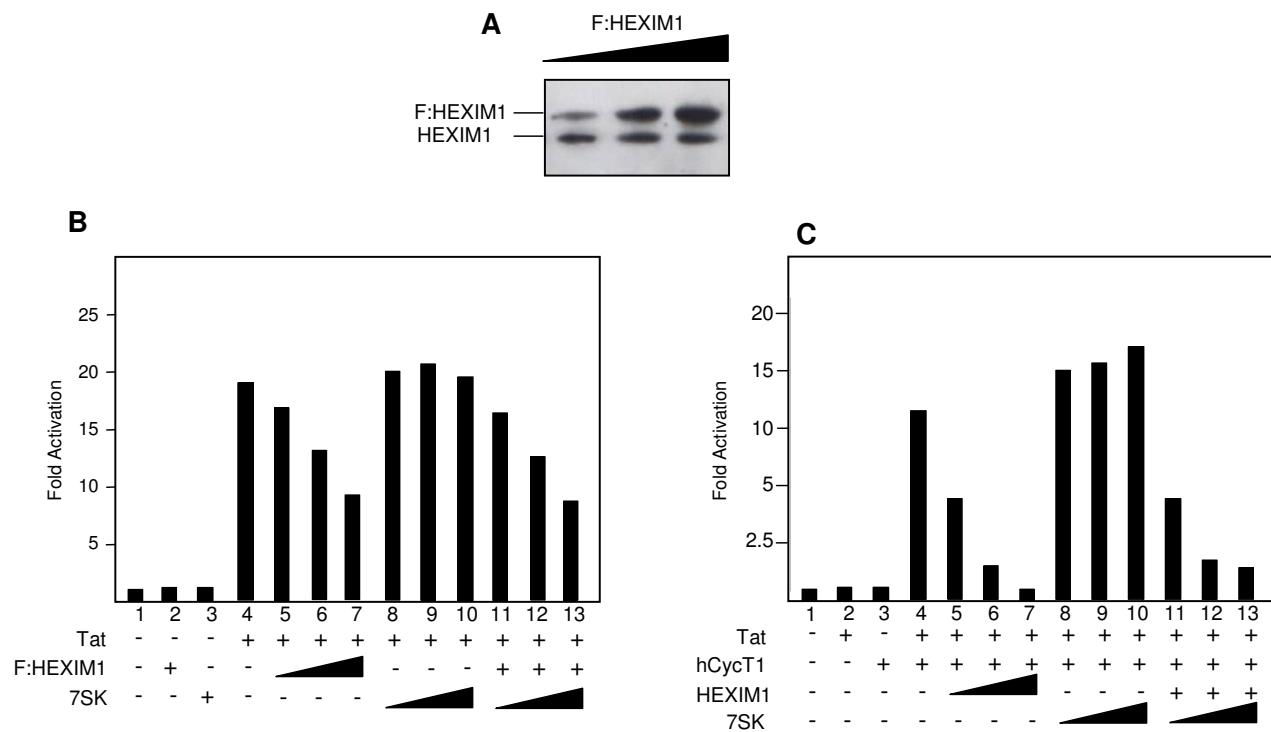


Figure 1
Overexpression of HEXIM1 protein represses Tat transactivation. Panel A, Increasing amounts (10, 100 and 500 ng) of Flag-tagged HEXIM1 were transfected into 293, cellular extracts were prepared at 48 hr after transfection and the relative levels on endogenous and exogenous HEXIM1 proteins were visualized by immunoblotting with anti-HEXIM1 antibody. Panel B, the HIV-Luc reporter (50 ng) was transfected into 293 cells in the presence of pSV-tat (50 ng) along with increasing (10, 100 and 500 ng) amounts of F:HEXIM1 and 7SK RNA (10, 100 and 500 ng), as indicated. Panel C, HEXIM1 decreases the co-operative effect of CycT1 on Tat activation in rodent cells. Chinese hamster ovary cells (CHO) were transfected with the HIV-LTR-Luc reporter (50 ng) in the presence of pSV-Tat (100 ng), lane 1, and together with CMV-hCycT1 (200 ng), lane 2, in the presence of increasing amounts of F:HEXIM1 and 7SK RNA as in panel B. Each histogram bar represents the mean of at least three independent transfections after normalization to Renilla luciferase activity to correct for transfection efficiency with the activity of the reporter without effect set to one. Standard deviations were less than 10%.

inhibition. The specific effect of HEXIM1 expression was highlighted by the results shown in Fig. 2B. G5HIV-Luc reporter was activated by co-expression of a GAL4-TBP, and such activation was largely unaffected by co-expression of HEXIM1. Thus, it appears that while HEXIM1 represses P-TEFb activity, enforced expression of this protein does not have significant effects on TBP-mediated basal transcription.

Definition of the HEXIM1 regulatory domains involved in repression
To investigate the structural determinants of HEXIM1 protein in repression, the activity of Gal4-CycT1 on G5HIV-

Luc was monitored in the presence of co-transfected Flag-tagged deletion mutants of HEXIM1. We found that removal of the C-terminal amino acids affected the inhibition as shown by the HEXIM1 (1-300) and (1-240) mutants (Figure 3 lanes 6-8 and 9-11). In contrast, removal of the 119 N-terminal amino acids of HEXIM1 (120-359) did not abolished inhibition (lanes 12-14). However, further deletion of the N-terminal amino acids (181-359) completely abolished the inhibitory effect (lanes 15-17). Thus, HEXIM1-mediated repression required the presence of the C-terminal domain (300-359aa) as well as a central region between residues 120 and 181. Finally, we found that HEXIM2, which like

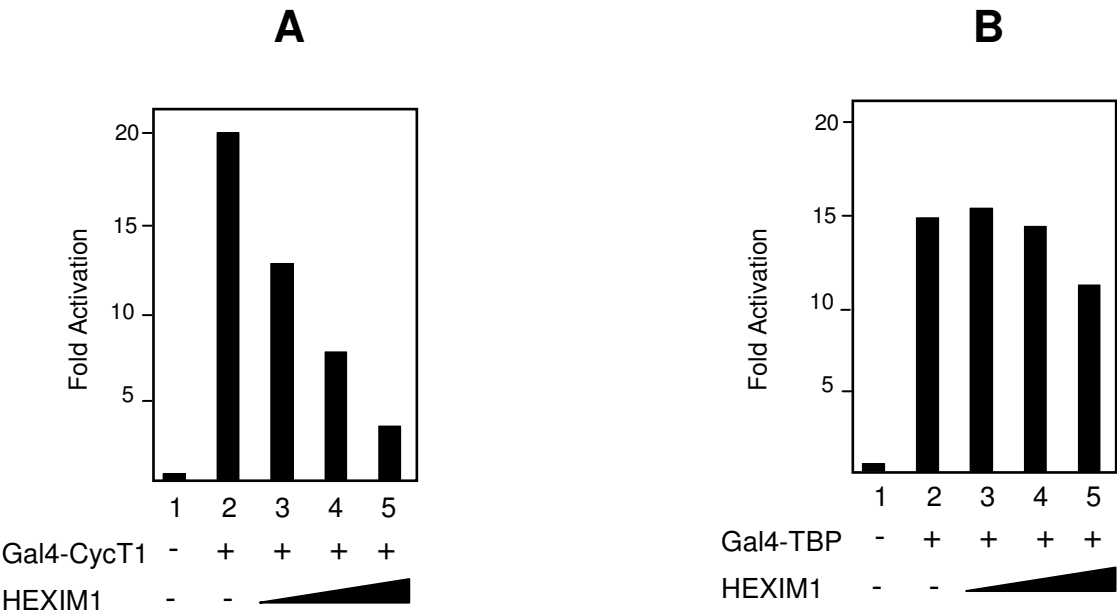
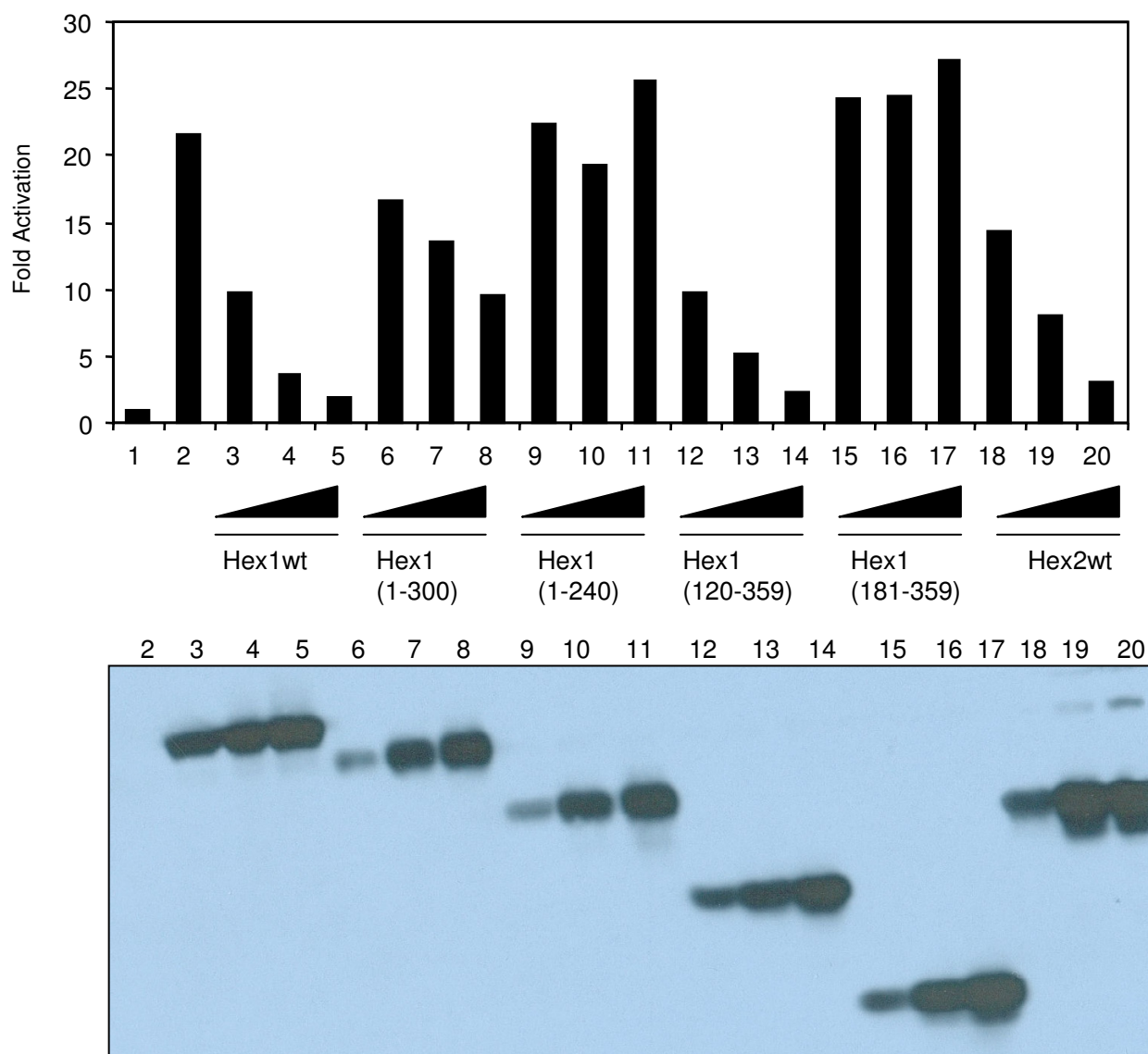


Figure 2
HEXIM1 represses GAL4-CycT1-mediated activation. Human 293 cells were transfected with 50 ng of G5-HIV Luc reporter DNA alone (lane 1) or in the presence of GAL4-expression plasmid DNA (200 ng), as indicated. The presence of the cotransfected F:HEXIM1 (10, 100 and 500 ng) is indicated. Each histogram bar represents the mean of three independent transfections after normalization to Renilla luciferase activity. The results are presented as described in figure 1.

HEXIM1, associates and inhibits P-TEFb activity, represses Gal4-CycT1 activation in a dose dependent manner (lanes 18–20).

We have recently reported that the HEXIM1 C-terminal domain (181–359) is involved in the binding to P-TEFb through direct interaction with the cyclin-box of cyclinT1 [15], and the evolutionarily conserved motif (PYNT aa 202–205) is important for such interaction. The PYND point mutant is impaired in repression and binding either P-TEFb or 7SK RNA in vivo, albeit it retains the ability to bind 7SK in vitro. In addition, we determined that

HEXIM1 binds 7SK snRNA directly and the RNA-recognition motif (KHRR) was identified in the central region of the protein (aa 152–155). In fact, the HEXIM1-ILAA mutant fails to interact in vivo and in vitro with 7SK snRNA [15]. To test the importance of these motifs in HEXIM1-mediated repression of Tat activity, HEXIM1 point mutants were co-transfected in 293 cells along with Tat or Gal4-CycT1, respectively. As shown in Figure 4, unlike wild-type HEXIM1, both mutants were unable to repress Tat as well as Gal4-CycT activities, albeit they were expressed at levels comparable to the wild-type protein. Collectively, the results presented in figures 3 and 4

**Figure 3**

HEXIM1 regulatory domains involved in repression. Human 293 cells were transfected with 50 ng of G5-HIV Luc reporter DNA alone (lane 1) or in the presence of 50 ng of pSV-Tat (lanes 2–20). The presence of increasing amounts (10, 100 and 500 ng) F:HEXIM1 wild-type (lanes 3–5), various deletion mutants (lanes 6–17) and F:HEXIM2 wt (18–20) are indicated, respectively. On the bottom, it is shown the western-blot of whole cells extracts from transfected cells probed with anti-Flag antibody from the indicated co-transfections. The results presented are from a single experiment after normalization to Renilla luciferase activity with the activity of the reporter without effect set to one. This experiment was performed three times with similar results.

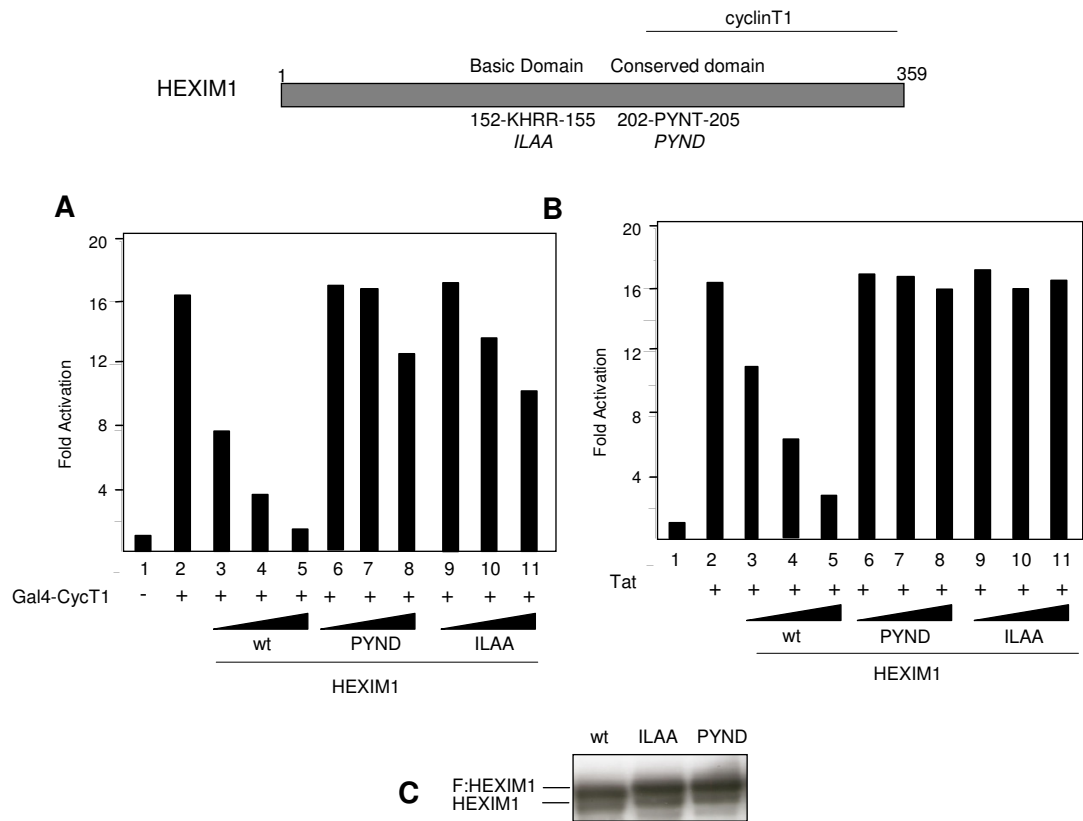
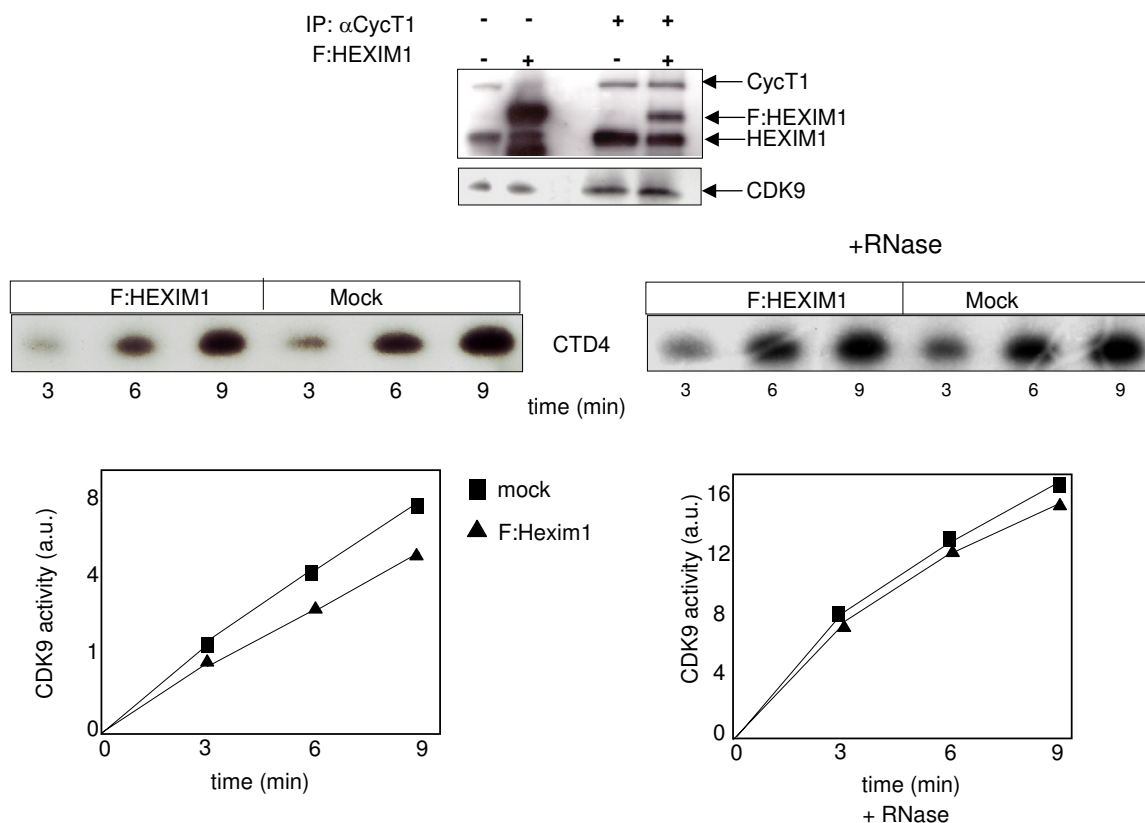


Figure 4
On top the relevant HEXIM1 functional domains are depicted. Position of the point mutants ILAA and PYND are indicated. G5-HIVLuc reporter (50 ng) was transfected into 293 cells along with Gal4-CycT I (200 ng) Panel A, or pSV-Tat (50 ng) panel B along with increasing amounts of Flag:HEXIM1 wilt type and mutants (10, 100 and 500 ng) as indicated. Each histogram bar represents the mean of three independent transfections after normalization to Renilla luciferase activity. The results are presented as described in figure 1. Panel C, western-blot with anti-HEXIM1 antibody demonstrated that the HEXIM1 effectors were expressed at comparable levels.

strongly suggest that HEXIM1-mediated inhibition of Tat activity requires interaction with P-TEFb as well as binding to 7SK snRNA.

P-TEFb activity in the presence of enhanced expression of HEXIM1

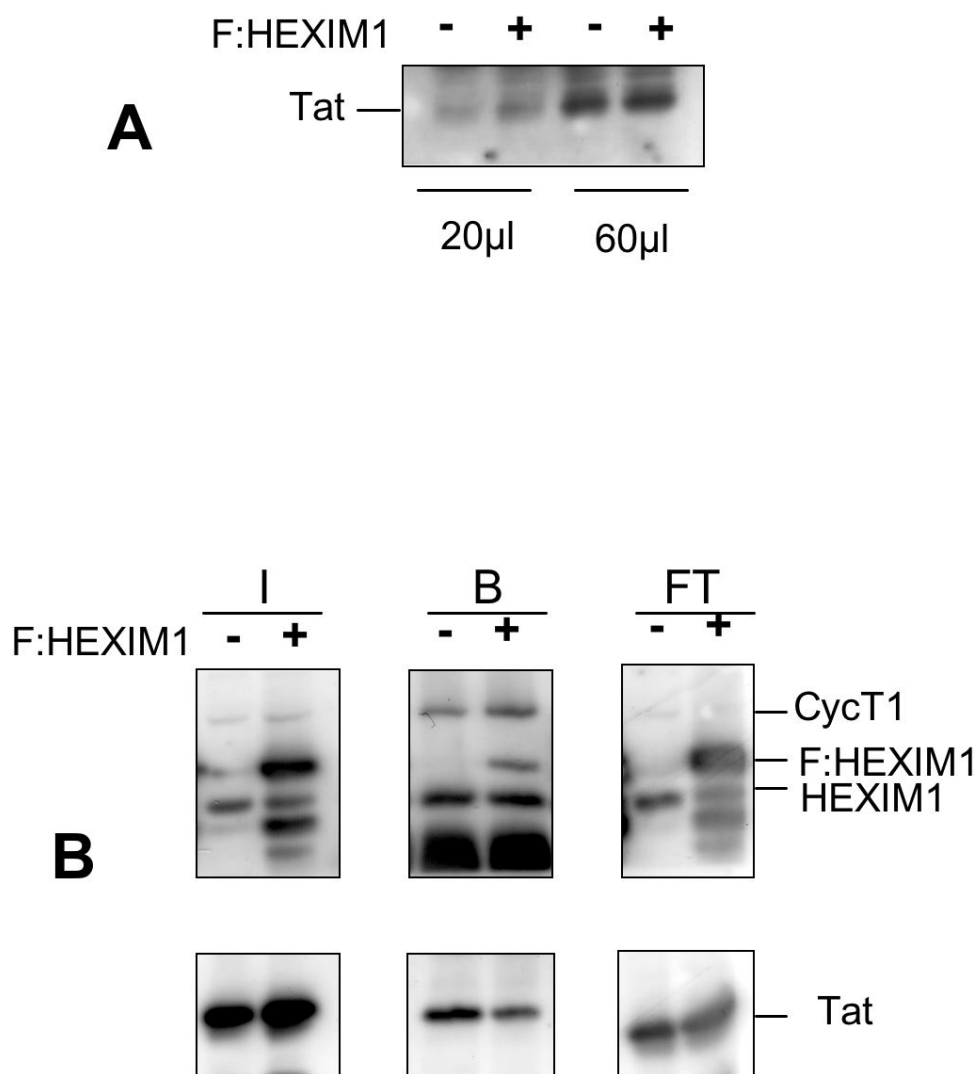
Next we sought to determine whether enhanced expression of HEXIM1 might directly affect the P-TEFb activity. 293 cells were transfected with F:HEXIM1 and cellular extracts from mock and transfected cells were prepared. P-TEFb activity was assayed using as substrate the CTD4 peptide consisting of four consensus repeats of the RNAPII CTD, and time-course kinase assays were performed [15]. Briefly, P-TEFb and its associated factors were affinity purified with anti-CycT1 antibody from mock and F:HEXIM1 transfected cell extracts. Immunoprecipitates were analyzed by immunoblotting for evaluation of CDK9, cyclin T1 and HEXIM1 proteins, respectively. The immunoprecipitates were then treated or not treated with RNase A (Fig. 5). The RNase treatment will degrade the 7SK snRNA thereby relieving the P-TEFb inhibition by HEXIM1/7SK snRNP. In fact, samples treated with RNase showed a robust increase in kinase activity compared those not treated with RNase,

**Figure 5**

P-TEFb activity in F:HEXIM1 transfected cells. Human 293 cells were transfected with 100 ng of F:HEXIM1 and cell extracts were prepared from mock and F:HEXIM1 expressing cells at 48 hr after transfection. Cell extracts were immunoprecipitated with anti-cycT1 antisera. The relative amounts of immunoprecipitated cyclinT1, CDK9 and HEXIM1 were quantitated by immunoblotting. Samples were treated or not treated with RNase, as indicated. Kinase assays were performed using a CTD4 peptide and ^{32}P incorporation was quantified in arbitrary units and plotted versus time (min). This experiment was performed four times with similar results. A typical experiment is shown.

indicating that 7SK snRNA had been effectively degraded. We found that the kinase activities of samples that were treated with RNase were quantitatively the same in both mock and F:HEXIM1 transfected extracts indicating equal amounts total of P-TEFb in both samples. A modest, but reproducible reduction of P-TEFb kinase activity (2-fold) was observed in extracts from F:HEXIM1 transfected cells. Altogether, these results demonstrated that over-expression of HEXIM1 resulted in a modest reduction of P-TEFb activity, thus the inhibition of Tat activity is unlikely due to a global reduction of cellular P-TEFb activity.

To further investigate the mechanism of inhibition of Tat-mediated transcription by HEXIM1, we tested the relative levels of transfected Tat protein in the presence of F:HEXIM1. We found that ectopic expression of HEXIM1 did not affect Tat expression (Figure 6A). Next, we sought to determine whether exogenous expression of HEXIM1 might result in a decrease in Tat-bound CycT1. To this end 293 cells were transfected with pSV-Tat in the presence or absence of F:HEXIM1 using the same transfection conditions used in the Luciferase assays. Cells extracts were immunoprecipitated with CycT1 antibody and the

**Figure 6**

Tat-CyclinT1 binding in the presence of HEXIM1. Panel A. 293 cells were transfected with 50 ng of pSV-Tat in the presence or absence of F:HEXIM1 (100 ng) as indicated and at 48 hrs after transfection cell extracts were probed by Western blotting with anti-Tat. For accurate comparison increasing amounts of material (µl) were loaded on the gels. Panel B. 293 cells were transfected as in Panel A, and cell extracts were immunoprecipitated with anti-CycT1. Immunocomplexes were analyzed on Western blots as indicated. I, input; B, bound; FT, flow through. This experiment was performed two times with similar results.

immunoprecipitates were analyzed by immunoblotting for evaluation of Tat, CycT1 and HEXIM1 proteins, respectively. In two different experiments we found a modest, but reproducible decrease in Tat-bound cyclin T1 (Fig. 6B). Thus, it appears that exogenous expression of HEXIM1 results in a decrease of Tat-bound P-TEFb.

Discussion

Several lines of evidence have suggested that Tat function is largely dependent upon the physical and functional interaction with the cellular transcription factor P-TEFb. The recruitment of P-TEFb to the LTR, involves the formation of the Tat-P-TEFb complex which efficiently binds TAR, allowing CDK9 to phosphorylate the CTD of RNAPII, thereby, enhances processivity of the polymerase to produce full-length mRNAs [6-10]. Two different P-

TEFb complexes exist *in vivo*. The core active P-TEFb comprises two subunits, the catalytic CDK9 and a regulatory partner cyclin T, and a larger inactive P-TEFb complex comprised by CDK9, cyclin T, HEXIM1 protein and the 7SK snRNA [11-17]. The relative presence of core and inactive P-TEFb complexes changes rapidly *in vivo* [11]. We have previously shown that the presence of HEXIM1/7SK snRNA in P-TEFb complexes prevents Tat binding to P-TEFb [13]. Since the association between 7SK RNA/HEXIM1 and P-TEFb competes with binding of Tat to cyclinT1, we have speculated that the TAR RNA/Tat system may compete with the cellular 7SK snRNA/HEXIM1 system [13]. Accordingly, it has been shown that over-expression of HEXIM1 represses Tat function [14,19]. We show here that HEXIM1 inhibits Tat function, while expression of 7SK snRNA does not influence Tat activity. It is pertinent to note that 7SK RNA is an abundant snRNA [23], and it is unlikely that 7SK might be rate-limiting for the assembly of the inactive P-TEFb complex.

We have delineated important structural domains of HEXIM1 required for repression of Tat. First, we found that the C-terminal region is required for inhibition. Previous findings indicated that the C-terminal region of HEXIM1 is involved in binding with cyclinT1 as well as for homo and hetero-dimerization with HEXIM2 [15,18,19]. Second, point mutations in the evolutionarily conserved motif PYNT (aa 202-205) abolished inhibition. It has recently shown a critical role of threonine 205 in P-TEFb binding [15]. Moreover, deletion mutants unable to bind P-TEFb failed to repress Tat (Figure 3). Therefore, it appears that HEXIM1 inhibition is strictly dependent upon the integrity of the protein to interact with P-TEFb. Third, a point mutant in the central part of HEXIM1 (KHRR motif aa 152-155) strongly affects Tat repression. Since this basic motif has been previously shown as the 7SK snRNA recognition motif [15], we conclude that interaction between HEXIM1 and 7SK snRNA is required for Tat repression. Collectively, these findings strongly suggested that HEXIM1-mediated inhibition of Tat required the formation of the P-TEFb/HEXIM1/7SK complex.

We determined that enhanced expression of HEXIM1 resulted in a modest inhibition (2-fold) of P-TEFb activity *in vivo*. Thus, HEXIM1-mediated inhibition of Tat activity is unlikely due to a global inhibition of P-TEFb activity. Moreover, we found that basal transcription from the LTR sequences was largely unaffected by over-expression of HEXIM1. Finally, ectopic expression of this protein does not have significant effects on TBP-mediated basal transcription. Thus, it appears that P-TEFb is specifically required for Tat-dependent HIV LTR transcription. Our results differ somewhat from those obtained in the Zhou lab who found that exogenous expression of HEXIM1

affects both basal as well as Tat-induced transcription [13]. These apparent discrepancies are possible due to different transfection conditions in which the relative amounts of the over-expressed exogenous proteins are likely different. We found that Tat expression which is under the control of SV40 promoter remains largely unaffected by co-expression of HEXIM. Our findings suggest a dedicated role of P-TEFb in Tat activity. Recent studies point to a specific role of P-TEFb for certain promoters. It has recently found that P-TEFb is recruited to the IL-8 but not to the I κ B α promoter [23], and it also represses transcription of regulators such as the nuclear receptor coactivator, PGC-1, in cardiac myocytes [24]. The specific HEXIM-mediated inhibition of Tat activity underlines the pivotal role of P-TEFb in the HIV LTR transcription.

The repression exerted by the HEXIM1 protein is likely the results of a competition between Tat and HEXIM1 in binding the P-TEFb. Since Tat binds only to the active P-TEFb complex, it has been suggested that Tat might trap the active form of P-TEFb as the PTEFb/7SK RNA/HEXIM1 complex appears to undergo continuous formation and disruption *in vivo*. In this scenario over expression of HEXIM1 may counteract the binding of Tat to P-TEFb, through a competitive association between the ectopic expressed HEXIM1 and P-TEFb. Accordingly, we found that exogenous expression of HEXIM1 results in a small but detectable reduction in Tat-bound P-TEFb. Our co-immunoprecipitation results are consistent with recent findings showing a mutually exclusive interaction of HEXIM1 and Tat with cyclinT1 using recombinant purified proteins [25]. Because Tat and HEXIM1 interact with the cyclin-box region of cyclinT1, it is plausible if not likely, that the mutually exclusive interaction of these two molecules with cyclinT1 is due to binding to the same domain or to a sterical hindrance. However, these studies have been performed *in vitro* in the absence of 7SK snRNA.

The results reported here along with previous findings strongly suggest the crucial role of 7SK in the interaction between HEXIM1 and cyclinT1. In fact, HEXIM1 ILAA mutant does not associate with 7SK *in vivo* and *in vitro*, and co-immunoprecipitation of cyclinT1 and 7SK RNA was markedly reduced with ILAA mutant compared to wild type [15]. Finally, as shown here ILAA mutant failed to repress Tat activity, suggesting an important role of HEXIM1/7SK interaction in Tat inhibition. Thus, association between HEXIM1 and 7SK snRNA appears an important determinant for Tat inhibition. Future *in vitro* and *in vivo* interaction studies, in the presence of 7SK snRNA may be instrumental to elucidate the role of 7SK/HEXIM1 complex in Tat activity.

Conclusion

The studies described in this provides further support to the pivotal role of P-TEFb for the optimal transcription Tat activity and highlight the importance of the P-TEFb cellular co-factors HEXIM1/7SK snRNA complex in Tat activity.

Methods

Tissue culture and transfections

Human 293 and rodent CHO cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco, Life Technologies). Subconfluent cell cultures were transfected cell cultures were transfected by a liposome method (LipofectAMINE reagent; Life Technologies, Inc.) in 2 cm/dish in multiwells, using 100 ng of reporter DNA and different amounts of activator plasmid DNA as indicated in the text and 20 ng of Renilla luciferase expression plasmid (pRL-CMV, Promega) for normalization of transfections efficiencies. Cells were harvested 48 h after DNA transfections, and cellular extracts were assayed for luciferase activity using Dual-Luciferase Reporter assay (Promega) according to the manufacturer's instructions. The experimental reporter luciferase activity was normalized to transfection efficiency as measured by the activity deriving from pRL-CMV.

Plasmids

The G5HIV-Luc contained the HIV-1 LTR sequences from -83 to +82 of LTR driven the Luc gene with 5 GAL4 DNA-binding sites inserted at -83. The pSV-Tat, GAL4-TBP, GAL4-CycT1, have been described [20]. 7SK snRNA plasmid was kindly provided by S. Murphy [22]. All Flag-tagged HEXIM1 and HEXIM2 expression vectors were constructed by insertion of the corresponding cDNA regions into the EcoRV site of p3xFlag-CMV10 vector (Clontech). Description of the deletion and point HEXIM1 mutants have been described previously [15]. Full description of the expression vectors used in this work is available upon request.

Western blotting and antibodies

Cells were lysed in ice-chilled buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 200 mM NaCl, 0.2 mM EDTA), supplemented with 1 mM dithiothreitol, 40 U/ml of RNasin (Promega), protease inhibitor cocktail (P-8340; Sigma), and 0.5 % Nonidet P-40. Lysates were vortexed and incubated for 20 min on ice and clarified by centrifugations. Western blottings were performed using the following antibodies: the rabbit polyclonal anti-HEXIM1 (C4) has been previously described (6); anti-FLAG M2 Monoclonal Antibody (Sigma), goat polyclonal anti-CycT1 (T-18), rabbit polyclonal anti-CDK9 (H-169) from Santa Cruz, anti-Tat (NIH AIDS Research Reagent Program). Binding was visualized by enhanced chemiluminescence (ECL-plus Kit, Amersham Biosciences).

Co-immunoprecipitation and kinase assay

293 cells were transfected with pSV-Tat in the presence or absence of F:HEXIM1 and cell extracts were prepared at 48 hrs after transfection. CycT1 was immunopurified from cell extracts (1 mg) using anti-CycT1 (H-245) (sc-10750, Santa Cruz). Input, immunoprecipitated and flow through materials were used in western blottings using anti-cycT1, anti-HEXIM1 and anti-Tat, respectively. For kinase assays 293 cells were transfected with F:HEXIM1 and after 48 hr P-TEFb complex was immunopurified from cell extracts (1 mg) using anti-CycT1 (H-245) (sc-10750, Santa Cruz) as previously described [13,15]. Briefly, whole cell extracts from mock and F:HEXIM1 transfected 293 cells were used in immunoprecipitations together with 40 µl of slurry beads (protein G-Sepharose 4 Fast Flow, Amersham Biosciences) pre-adsorbed with anti-CycT1 and the interactions were carried out in buffer A for one hour at 4°C on a wheel. After extensive washes one half of the immunopurified materials was used in western blotting to ensure the presence of equal amounts of CDK9, HEXIM1 and CycT1, respectively. The remaining material was suspended and stirred at room temperature and split in two equal aliquots. One of the aliquot was treated with 10U of RNase A for 15 min at 30°C. Samples treated or not with RNase were stirred at room temperature for three minutes in 65 µl of buffer A containing [γ -³²P]ATP (0,1 µCi/µl), 40 mM ATP, 0,1 µg/ml (YSPTSPS)₄ peptide CTD4 (6, 8) and RNasin (40 U/ml). Aliquots (20 µl) of the suspension were mixed with SDS-PAGE loading buffer at intervals of three minutes to stop the reaction. The phosphorylated CTD4 substrate was separated on a 15% SDS-PAGE and visualized by radiography. Incorporation of [³²P] into CTD peptide was quantified on a Bio-Rad phosphorimager.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AF carried out the transfection studies and plasmid construction. FV performed studies using the HEXIM1 point mutants. GN carried out the kinase experiments. AAM isolated and constructed the HEXIM2 expression vector. BM and OB participated on discussion of results and drafting the manuscript. LL designed this study and edited the manuscript.

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Research Paper

p14ARF Directly Interacts with Myc Through the Myc BoxII Domain

Stefano Amente¹

Barbara Gargano¹

Francesca Varrone¹

Luca Ruggiero^{1,2}

David Dominguez-Sola²

Luigi Lania¹

Barbara Majello^{1,*}

¹Department of Structural and Functional Biology; University of Naples 'Federico II'; Naples, Italy.

²Institute for Cancer Genetics; New York, New York USA

*Correspondence to: Barbara Majello; Department of Structural and Functional Biology; University of Naples 'Federico II'; Naples, Italy; Tel.: +39.081.679062; Fax: +39.081.679233; Email: majello@unina.it

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KEY WORDS

Myc, Myc p14ARF, Myc BoxII, protein interactions, transcription, tumor suppressor

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ABSTRACT

Myc is a well known proto-oncogene encoding for a transcription factor whose activity is tightly regulated in the cellular context. Myc was the first oncogene recognized to activate the ARF tumor suppressor gene which suppresses cell proliferation partly through stabilization of the p53 tumor suppressor protein but which also has p53-independent growth-suppressive functions. Recent studies have indicated that mouse p19ARF negatively regulates Myc's transcriptional activity. We here show that the human p14ARF directly associates with Myc and relocates Myc from the nucleoplasm to the nucleolus. We found that p14ARF interacts with the Myc-Max complex and the binding of p14ARF does not interfere with Myc-Max interaction in vitro. Protein interaction assays define the Myc BoxII as a critical domain required for interaction with p14ARF. Moreover, we identify 30 amino acids encompassing Myc BoxII domain required for p14ARF interaction and colocalization in vivo. Finally, we show that p14ARF down regulates Myc activated transcription and that this activity cannot be addressed to an intrinsic p14ARF repressor domain.

INTRODUCTION

Deregulated expression of Myc protein contributes to several aspect of tumor cell biology. Enhanced expression of Myc can drive unrestricted cell proliferation, inhibition of cell differentiation and vasculogenesis, reduce cell adhesion, promotes metastasis and genomic instability. Conversely loss of Myc protein not only inhibits cell proliferation and growth, but can also increase cell differentiation and adhesion.^{1,2,3} High levels of Myc activity are required for ARF induction and Myc enhancing effect on cell proliferation is inhibited by the ARF-Mdm2-p53 pathway.^{4,5}

The Myc protein contains a transactivation domain (TAD) at the amino terminus and a basic region directly followed by a basic helix loop helix leucine zipper motif (bHLH-LZ) at its carboxyl terminus. The bHLH-LZ domain is responsible for DNA binding and heterodimerization with partner proteins. Myc partner is the Max protein that also has a bHLH-LZ domain through which forms a Myc-Max complex that specifically binds to E-box sequences on gene targeted promoters. The TAD of Myc contains two regions, Myc BoxI and II that are highly conserved in sequence among Myc family members. Both of these regions appear fundamental for optimal transcriptional transactivation.³

On the other end, in a context independent from the E-box recruitment, high levels of Myc have been correlated with transcriptional repression of a number of genes including p15 and p21.^{1,6} A regulated transition between the activating and repressing role of the Myc protein might depend from the physiological status of the cell. The ARF protein has been recently found to associate with Myc independently from p53 and to inhibit transcription but not repression by Myc.^{4,7,8}

The ARF tumor suppressor (p19ARF in mouse or p14ARF in human) antagonizes the occurrence of various tumors. ARF up-regulates the p53 tumor-suppressing activity by interacting with Mdm2 and increasing protein expression of p53.^{9,10} In addition, several evidences support the involvement of ARF in the inhibition of cell proliferation independently of p53.^{10,11} Mouse and human ARF proteins share a limited homology at the cDNA and protein levels (132aa for p14ARF versus 169aa for p19ARF).¹¹ These differences at the structural level have consequences whose functional relevance still is obscure. Different partners, according to the species, have been found, while p19ARF is able to interact with 5.8S rRNA, the p14 human protein has never been found in a 5.8S rRNA complex.^{12,13}

The structural differences between the murine p19ARF and the human p14ARF proteins led us to investigate if also the human p14ARF tumor suppressor protein associated with the Myc protein and if this association was a direct binding between the two proteins.

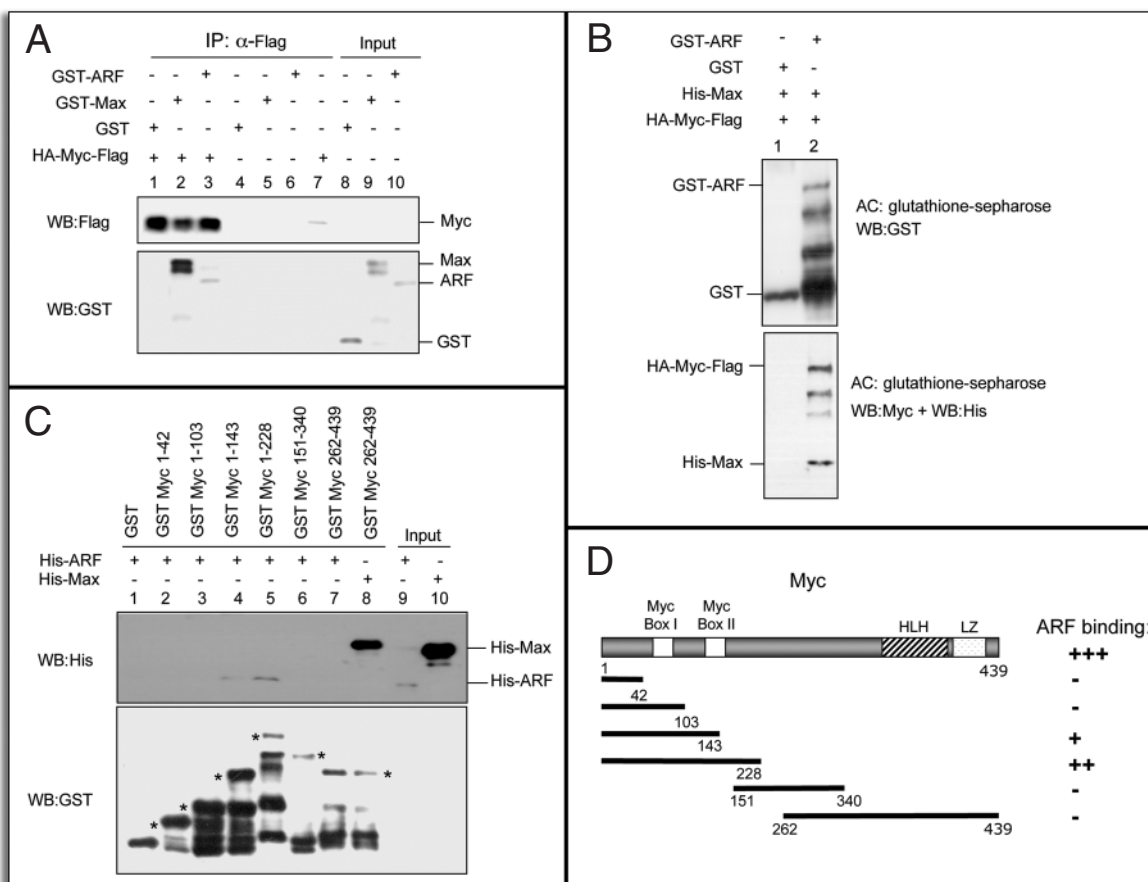


Figure 1. p14ARF directly interacts with Myc in vitro. In the in vitro pull-down assays all the GST-fusions were purified using glutathione-sepharose and subsequently eluted from the beads by 20 mM glutathione incubation. The His-Max and His-p14ARF proteins were affinity purified by using Ni-NTA Agarose (Invitrogen life technologies) and subsequently eluted according to the manufacturer's instructions. The HA-Myc-FLAG protein was double purified first with ANTI-FLAG M2-Agarose Affinity Gel (Sigma) followed with Monoclonal ANTI-HA Agarose Conjugate (Sigma). (A) The GST, GST-Max or GST-p14ARF proteins (600 ng) were incubated in presence (lanes 1–3) or absence (lanes 4–6) of HA-Myc-FLAG bait protein (600 ng). Protein complexes were recovered by immunoaffinity with ANTI-FLAG M2-Agarose and analyzed by WB with anti-FLAG (top panel) and anti-GST (bottom panel) (Santa Cruz Biotechnology, Inc.). In lanes 8, 9, 10 and 5% of the inputs were loaded. (B) GST-p14ARF and GST-Max proteins (600 ng) were incubated with equal amounts of GST (lane 1) or HA-Myc-FLAG (lane 2). Affinity complexes (AC) were analyzed by WB using anti-GST (top panel) and a mixture of anti-Myc plus 6xHis Monoclonal Antibody (BD Biosciences, bottom panel). (C) Mapping of the Myc domains involved in ARF interaction. His-p14ARF was incubated with GST and GST-Myc deletion mutants (lanes 1–7) proteins. In lane 8, as positive control of Myc interaction, GST-Myc 262–439 was incubated with the His-Max. The protein complexes were recovered using glutathione-sepharose and the copurified proteins revealed with anti-His (top panel) and anti-GST (bottom panel). In lane 9 and 10, 5% of the proteins inputs were loaded. (D) Schematic representation of the Myc full-length protein and deletion mutants. The relative strengths of interactions with p14ARF is indicated.

MATERIALS AND METHODS

Plasmids. GAL4-KRAB and G5-83HIV-Luc plasmids have been previously described.¹⁴ Gal4-p14ARF was constructed by inserting an EcoRI/SalI fragment containing the ARF cDNA obtained from the GFP-p14ARF into the pSG424 vector. The same insert was subcloned in pPROEX HTa vector (GIBCO Life Technologies) to obtain the pHIS-ARF vector. GFP-p14ARF, GST-p14ARE, GST-Max, His-Max, pcDNA3-Max, pHA-Myc-FLAG, pcDNA3-FLAG-Myc, pcDNA3-FLAG-Myc Δ 123–151, pMT2T-Myc, pcDNA-p14ARF-HA, GST-Myc deletion mutants, hTERT-Luc were kindly provided by G. La Mantia and R. Dalla Favera.

In vitro proteins binding assays. Bacterial cells were lysed in PBS 1X Buffer with 1mM PMSF and protease inhibitors and subsequently sonicated. The lysates were centrifuged and recombinant proteins were affinity purified: the GST-fusions (GST; GST-ARF; GST-Max; GST-Myc 1–42; GST-Myc 1–103; GST-Myc 1–143; GST-Myc 1–228; GST-Myc 151–340; GST-Myc 262–349) were purified as previously described¹⁵ using glutathione-sepharose (Amersham Biosciences) and subsequently eluted from the beads

by 20 mM glutathione incubation. The His-Max and His-ARF proteins were affinity purified by using Ni-NTA Agarose (Invitrogen life technologies) and subsequently eluted in Buffer C (20 mM Tris-HCl; 100 mM KCl; 5 mM 2-mercaptoethanol, 10% glycerol, 100mM imidazole). The HA-Myc-FLAG protein was double purified in two steps: in the first step the recombinant protein was affinity purified with ANTI-FLAG M2-Agarose Affinity Gel (Sigma) and subsequently eluted by incubation with the FLAG peptide; in the second step the resulting protein was incubated with Monoclonal Anti-HA Agarose Conjugate (Sigma) and eluted from these beads by using HA Peptide (Sigma). For the individual experiments 600 ng of each recombinant protein were incubated in a final volume of 1 ml of Binding Buffer (50 mM Tris-HCl pH 7.4; 150–500 mM NaCl; 1 mM MgCl₂; 1 mM DTT; 0.2% NP40). After extensive washing in Binding Buffer, the bound proteins were eluted by 2X Laemmli buffer, separated on SDS PAGE followed by Western Blotting.

Cell culture, Luciferase assays and immunofluorescence. Human 293T and U2OS cell lines were grown in DMEM supplemented with 10% fetal calf serum. For the luciferase assay, cells were transfected with lipofectamine

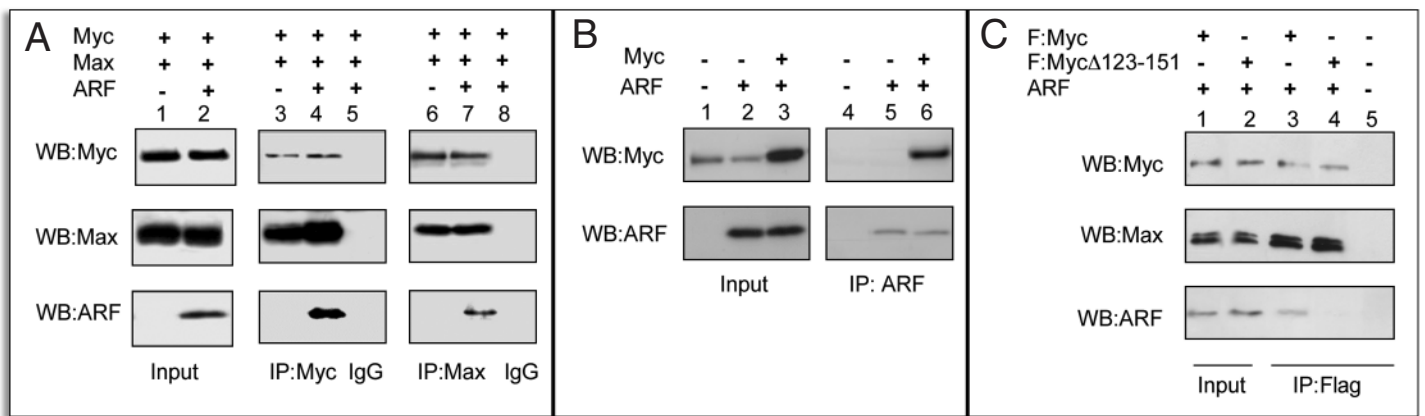


Figure 2. p14ARF interacts with Myc in vivo. (A) 293T cells were cotransfected by the calcium-phosphate method with of pcDNA3-FLAG-Myc, pcDNA3-Max and pcDNA-ARF-HA as indicated. Protein extracts were immunoprecipitated with the anti-Myc N262 antibody (lanes 3–5), anti-Max antibody (Santa Cruz Biotechnology, Inc; lanes 6–8), and IgG antibody (lanes 5,8), and the copurified complexes analyzed by WB with anti-Myc 9E10, anti-Max and anti-ARF antibodies, as indicated. 5% of the proteins inputs were loaded in lanes 1 and 2. (B) U2OS cells were cotransfected with the indicated vectors and protein extracts were IP with anti-ARF antibody (lanes 4–6) and the CoIP complexes analyzed by WB with anti-Myc (top) and anti-ARF (bottom). 5% of the proteins inputs were loaded in lanes 1–3. (C) Myc BoxII is required for the Myc-ARF interaction in vivo. 293T cells were cotransfected with pcDNA-p14ARF-HA along with pcDNA3-FLAG-Myc or pcDNA3-FLAG-MycΔ123-151 indicated. Protein extracts from the transfected cells were IP with ANTI-FLAG M2-Agarose followed by WBs with anti-Myc (top), anti-Max (middle) and anti-ARF (bottom).

2000 (Invitrogen Technologies) and pRL-CMV (Promega) was cotransfected for normalization. The amounts of transfected plasmids DNAs are indicated in the legend to figures. For immunofluorescence analysis U2OS cells were transfected with lipofectamine 2000 with 200 ng of the pcDNA3-FLAG-Myc, pcDNA3-FLAG-MycΔ123-151, GAL4-KRAB, and GAL4-ARF plasmid and cells processed as described¹⁶ using the anti-GAL4 (Upstate Bio, Inc) and anti-Myc (9E10, Santa Cruz Biotechnology, Inc.) antibodies.

Antibodies and coimmunoprecipitations. The following antibodies were used for the immunological techniques: anti-Myc (N262 for IP and 9E10 for WB, Santa Cruz Biotechnology, Inc.), anti-Max (C17, Santa Cruz Biotechnology, Inc.), anti-ARF (C-18, Santa Cruz Biotechnology, Inc.), anti-FLAG M2 Monoclonal Antibody-Peroxidase Conjugate (Sigma), anti-GST (B-14, Santa Cruz Biotechnology, Inc.), 6xHis Monoclonal Antibody (BD Biosciences), anti-GST (B-14, Santa Cruz Biotechnology, Inc.), anti-Gal4 (Upstate Bio, Inc.). Coimmunoprecipitations from transiently transfected cells was carried out as previously described.¹⁵ For c-Myc IP, 5 µg of antibody or IgG plus 25 µl of preequilibrated protein G-Sepharose 4 Fast Flow (Amersham Biosciences) was used; for Max IP, 3 µg of antibody or IgG was used plus 25 µl of preequilibrated protein G-Sepharose 4 Fast Flow (Amersham Biosciences) was used. For FLAG IP, 25 µl of pre-equilibrated anti-FLAG M2-Agarose Affinity Gel (Sigma) was used. All interactions were carried out overnight at 4°C. Beads were washed at least five times using buffer F (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 30 mM Na₂O₇P₂, 50 mM NaF, 5 µM ZnCl₂, 0.1 mM Na₃VO₄, 1% Triton, 0.1 mM PMSF) before loading on SDS-PAGE.

RESULTS

p14ARF directly interacts with cMyc. Recent studies have indicated that mouse p19ARF interacts and negatively regulates Myc's transcriptional activity. The structural differences between the murine p19ARF and the human p14ARF proteins prompt us to determine if also the human p14ARF tumor suppressor protein associated with the human Myc protein. To this end we performed in vitro interaction assays using highly purified bacterial expressed HA-Myc-FLAG and GST-p14ARF proteins. Equal amounts of the two purified proteins were incubated for three hours and subsequently the HA-Myc-FLAG protein complex was recovered with M2-FLAG beads and analyzed by western blotting with the GST antibody for the presence of the GST-p14ARF copurified protein. In addition, the HA-Myc-FLAG was incubated with GST and GST-Max proteins as negative and positive interaction

controls, respectively (Fig. 1A, lane 1 and 2). The result showed in Figure 1A, demonstrates that Myc directly interacts with p14ARF (lane 3).

Interaction between Myc and p19ARF has been shown to alter the transcription activity of Myc.^{4,7,8} Since Myc binds to target promoters as heterodimer with Max, we sought to determine if p14ARF was able to bind to the Myc-Max heterodimer, and most importantly to determine a possible mutually exclusive interaction of p14ARF and Max with Myc. Purified GST-p14ARF, HA-Myc-FLAG and His-Max proteins were incubated and the GST-affinity complex was purified. The affinity-purified complex (AC) was then analyzed for the presence of Myc and Max proteins by immunoblotting with anti-Myc and anti-His antibodies, respectively. The result in Figure 1B, lane 2, shows that the GST-p14ARF pull-down both Myc and Max, demonstrating that p14ARF interacts with the Myc-Max complex and that the binding of p14ARF does not interfere with Myc-Max interaction in vitro.

Myc BoxII is required for Myc-ARF interaction in vivo and in vitro. To identify Myc protein sequences that are essential for association with p14ARF, we performed in vitro GST pull-down assays using the His-p14ARF protein and various GST-Myc deletion mutants. The different GST-Myc deletion mutants were mixed with the His-p14ARF protein and the complexes were affinity-purified by GST beads; the presence of p14ARF was monitored by immunoblotting with His antibody. Aliquots of each sample were assayed with the GST antibody for the presence of the different GST-Myc mutants used as baits. The results in Figure 1C show that the Myc deletion mutants 1-143 and 1-228 (lane 4 and 5) retain the ability to bind the His-p14ARF protein. In particular, the GST-Myc 1-228 shows stronger interaction. In contrast, the N-terminal deletion mutants, GST-Myc 151-340 and GST-Myc 262-439 fail to bind His-p14ARF (lanes 6 and 7). As positive control of interaction the GST-Myc 262-439 interacts with His-Max (lane 8). Thus, the Myc residues 103 to 151, including the Myc BoxII domain, are required for association with p14ARF in vitro.

To determine whether Myc/Max heterodimer interacts with p14ARF in vivo, CoIP assays were performed with protein extracts from transiently transfected 293T cells that express low levels of endogenous Myc and ARF proteins. The cells were transfected with CMV-based Myc and Max expression vectors in the presence and absence of p14ARF, and immunoprecipitations were performed using anti-Myc or anti-Max antibodies. Immunoblot analysis was then performed using ARF antibody. The results reported in Figure 2A show that either the p14ARF or Max proteins coimmunoprecipitated with Myc. In parallel the extracts were immunoprecipitated with the Max antibody

followed by immunoblotting with Myc, Max and ARF antibodies, respectively. As shown in Figure 2A, Max immunoprecipitated extracts contained both the Myc and the ARF proteins (lane 7). Collectively these findings demonstrate that p14ARF associates with Myc/Max heterodimer in vitro and in vivo and that Myc binding to Max and p14ARF is not mutually exclusive. To further validate the Myc-ARF interaction the U2OS cell line, which do not express the ARF protein, were transfected with CMV-based Myc and ARF expression vectors and cellular extracts were immunoprecipitated with the ARF antibody. Immunoblots confirmed the presence of the Myc protein in the immunoprecipitated extracts (Fig. 2B, lane 6).

To corroborate the requirement of the Myc BoxII in the interaction with the p14ARF, we performed CoIP assays with protein extracts prepared from 293T cells that were transiently cotransfected with a CMV driven p14ARF expression vector along with the pcDNA3-FLAG-Myc vector expressing the full-length protein or an isogenic vector, pcDNA3-FLAG-Myc Δ 123-151, expressing a protein with an in-frame deletion of the Myc BoxII domain. Protein extracts were immunoprecipitated with the FLAG antibody followed by immunoblot with the ARF and Max antibody, respectively. The result shown in Figure 2C, illustrate that while the full-length Myc protein interacts with both p14ARF and Max, the deletion of a 123 to 151, including the Myc BoxII, severely invalidates the Myc-ARF binding, without significant effects on Max binding. Collectively, these results substantiated the physical interaction between ARF and Myc and demonstrate that the region encompassing the Myc BoxII is involved in the interaction between Myc and p14ARF both in vitro and in vivo.

Myc-p14ARF nucleolar colocalization is abrogated by Myc BoxII deletion. The Myc protein localizes in the cellular nucleus while the p14ARF protein has a predominantly nucleolar localization. Depending from the cell line in which the Myc-ARF interaction has been investigated, it has been reported that, upon ectopic expression of ARF, Myc colocalizes with ARF in nucleoli.⁷ Consequently, we sought to analyze the contribution of the Myc BoxII region, required for in vivo and in vitro binding, in the sub-cellular colocalization of Myc and p14ARF. U2OS cells were cotransfected with a green fluorescent protein (GFP) fusion, GFP-p14ARF, along with the Myc expression vector or the deletion mutant Myc Δ 123-151. In agreement with previous observations, we found that the GFP-p14ARF accumulates predominantly into the nucleolus whereas Myc displayed a typical nucleoplasmic localization (data not shown). When ARF and Myc were cotransfected in a 1-to1 ratio, 82% of the cotransfected cells exhibited colocalization of Myc and ARF protein into the nucleoli (Fig. 3). In contrast, a significant reduction of colocalization into the nucleoli (22%) was observed when GFP-p14ARF was cotransfected with the Myc Δ 123-151 deletion mutant. These findings underlie the relevance of the Myc BoxII domain in the physiological interaction between the ARF and Myc protein.

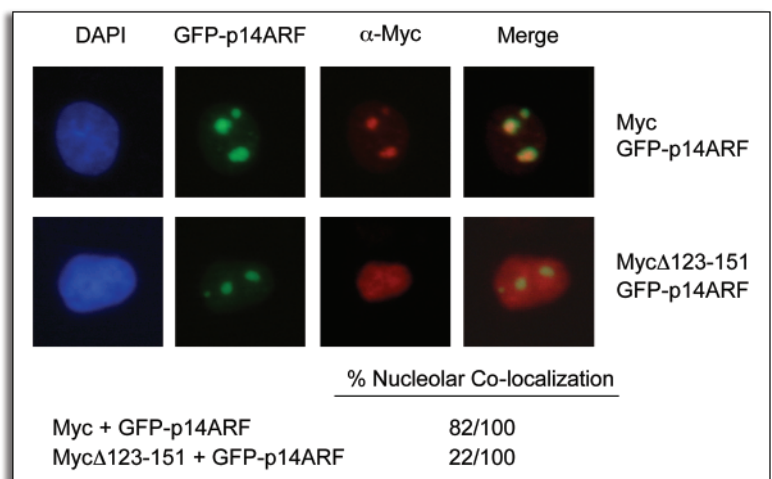
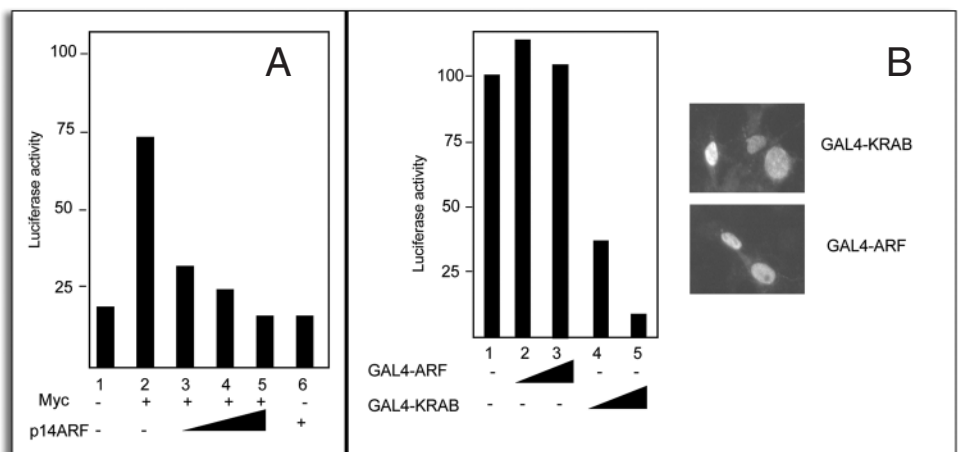


Figure 3. Myc-ARF nucleolar colocalization is impaired by Myc BoxII deletion. U2OS cells were cotransfected with GFP-p14ARF and pcDNA3-FLAG-Myc or pcDNA3-FLAG-Myc Δ 123-151 by Lipofectamine 2000 as indicated. An example of immunofluorescence microscopy of the cells immunostained with anti-Myc 9E10 and analyzed by fluorescence microscopy as previously described, is shown. At least 150 cells were analyzed in each experiment. Values are means from three independent experiments. All images were digitally processed using Adobe Photoshop.

It has been shown that mouse p19ARF is able to block Myc's ability to activate transcription.^{4,8} To extend these results to the human ARF homolog, we next investigated if p14ARF expression in the U2OS cell line was able to inhibit Myc ability to transactivate the Telomerase Reverse Transcriptase (hTERT) promoter.¹⁷ As reported in Figure 4A, Myc exogenous expression in U2OS cells activates the hTERT-Luc promoter expression and cotransfection of p14ARF inhibits Myc-activation in a dose dependent manner.

p14 ARF does not possess an intrinsic repressor domain. Several models can be envisaged to explain how ARF might repress Myc mediated transcription. For example ARF may possess an intrinsic repressor domain. In order to evaluate if the p14ARF protein is able to repress transcription when artificially recruited on a targeted promoter, the human ARF cDNA was fused to the yeast GAL4 DNA binding domain and the activity of the resulting plasmid, GAL4-p14ARF, assayed on a targeted promoter bearing 5xGAL4 DNA binding sequences. As control, the GAL4-KRAB expression vector,¹⁶ bearing the well described KRAB repressor domain was used. As shown in Figure 4B, artificial recruitment of GAL4-p14ARF has no significant effects on G5-83HIV-Luc expression, while GAL4-KRAB effectively repressed the G5-83HIV-Luc promoter's activity. Immunofluorescence of transfected cells with a GAL4 antibody, indicated that both GAL4 fusion proteins are

Figure 4. ARF expression down regulates Myc activated transcription of the human TERT promoter. (A) U2OS cells were cotransfected with 100 ng of hTERT-Luc, 200 ng pMT2T-Myc and different amounts (0.1; 0.5 and 1 μ g, respectively) of pcDNA-p14ARF-HA as indicated. (B) Left panel, GAL4-p14ARF fusion protein artificial recruitment. Cos cells were cotransfected by Lipofectamine with 100 ng the G5-83HIV-Luc vector, GAL4-p14ARF (0.5 and 1 μ g) and GAL4-KRAB (0.5 and 1 μ g) as indicated. In the right panel, the immunostaining with anti-GAL4 of transfected cells is shown. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%.



expressed at comparable levels (percentage of GAL4 positive cells) and both proteins display a prominent nuclear localization (Fig. 4B, right panel). Thus, p14ARF does not appear to possess an intrinsic repression capability.

DISCUSSION

It has been recently shown that mouse p19ARF negatively regulates Myc's transcriptional activity. Even though 14ARF and p19ARF are only 50% identical, their biological function, and interaction with Myc appears to be very similar. We show here by in vitro pull down assays and in vivo CoIPs that c-Myc binds directly to p14ARF and that Myc Box II is critical for the interaction. We also found that p14ARF does not interfere with the binding between c-Myc and Max. In addition, p14ARF inhibits the activation of the hTERT promoter by Myc, without having any intrinsic repression activity itself. While Myc protein is a nucleoplasmatic protein the p14ARF protein has a predominantly nucleolar localization. When both proteins were expressed we found that the majority of cotransfected cells exhibited colocalization of Myc and ARF protein into the nucleoli. Previous studies have shown that, depending from the cell line in which the Myc-ARF interaction has been investigated, upon ectopic expression of p19ARF, Myc colocalizes with p19ARF in nucleoli.⁷

It remains largely unknown how ARF downregulates Myc transcription activity. It is quite unlikely that ARF could affect recruitment of Myc-Max heterodimer on DNA target promoters. Gels retardation experiments indicated that expression of the p14ARF protein in the U2OS cells does not reduce the association of the Myc-Max complex to the target DNA sequence (Amente S, Gargano B, unpublished observation). Accordingly, ChIP analysis on Myc targeted promoters indicate that Myc binds to the eIF4E and nucleolin genes promoters whether or not p19ARF is present indicating that p19ARF does not affect recruitment of Myc on these target genes.⁸

Our findings strongly suggest the critical role of Myc BoxII in ARF interaction. In fact, we identify 30 amino acids encompassing Myc BoxII domain required for p14ARF interaction and colocalization in vivo. A large number of evidences demonstrated that Myc BoxII is required for activation and repression of most target genes.^{1,3} In addition to ARF, four proteins can bind directly to this region: the TRRAP, a core subunit of the TIP60 and GCN5 histone acetyltransferase complex (HAT),¹⁸ the ATPases TIP48 and TIP49 found in chromatin remodeling complexes,¹⁹ and the SKP2 protein involved in ubiquitilation.²⁰ However, it is not known if these proteins bind Myc simultaneously or Myc forms separate complexes with some of them. We speculate that ARF binding may alter the association of Myc to dedicated cofactors either through steric hindrance or to a mutually exclusive interaction due to binding to the same domain.

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Letter to the Editor

Activation of P-TEFb Induces p21 Leading to Cell Cycle Arrest

Giuliana Napolitano[†]

Francesca Varrone[†]

Barbara Majello

Luigi Lania*

Department of Structural and Functional Biology; University of Naples 'Federico II'; Naples, Italy

[†]These authors contributed equally to this work

*Correspondence to: Luigi Lania; Department of Structural and Functional Biology; University of Naples 'Federico II'; via Cinthia; 80126 Naples, Italy; Tel.: +39.081679064; Fax: +39.081679233; Email: lania@unina.it

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The positive transcription elongation factor (P-TEFb) is a cyclin-dependent kinase responsible for phosphorylation of the carboxyl-terminal-domain (CTD) of the larger subunit of RNA polymerase II as well as the negative elongation factors NELF and DSIF for a recent review (see ref. 1 and refs. therein). P-TEFb activity is controlled in vivo by negative as well as positive factors. In cultured HeLa cells two equivalent amounts of different P-TEFb complexes exist in vivo.²⁻⁵ The core active P-TEFb is a heterodimer composed of CDK9 and cyclin T1 or T2 (CycT1 and CycT2). P-TEFb is negatively regulated by HEXIM1, which in association with 7SK snRNA hold P-TEFb in a kinase-defective large 7SK-HEXIM1/P-TEFb snRNP complex.^{6,7}

Following cellular stress such as genotoxic insults, UV irradiation, cardiac hypertrophic stimuli or treatments that induce transcriptional arrest, 7SK and HEXIM1 dissociate from the ribonucleo-protein complex, causing the rapid accumulation of the catalytically active P-TEFb.^{2,3} In contrast with a plethora of positive P-TEFb cofactors, HEXIM1 is the only known negative regulator. HEXIM1 has the inherent ability to associate with P-TEFb and binding of 7SK snRNA turns the HEXIM1 into a P-TEFb inhibitor.^{6,7} HEXIM1 has been originally isolated as a protein which is induced in smooth muscle cells following exposure to Hexamethylene bisacetamide (HMBA). Moreover, HMBA treatment leads to enhanced expression of HEXIM1 in virtually all cell lines tested, and overexpression of HEXIM1 inhibits P-TEFb activity.^{5,8} Recently, we reported that HEXIM1 causes growth inhibition and promotes neuronal differentiation.⁹ Collectively, these findings provide circumstantial evidences on a putative role of HEXIM1 in cell proliferation.

HMBA is widely known to induce cell cycle arrest, differentiation and/or apoptosis in various cell types.^{10,11} However, it remains unclear whether these effects are mediated by functional variations of the HEXIM1/P-TEFb equilibrium. Here, we addressed this issue by analyzing the functional consequences of HMBA on P-TEFb activity and cell cycle progression.

We sought to determine changes in the cell cycle profile of a clonal HeLa cell line (huHL6) bearing an integrated HIV-LTR-Luc reporter, exposed to HMBA. As shown in Figure 1A, treatment of huHL6 cells with HMBA induced a G₁ arrest. Consistent with this observation, western blot analysis revealed a concomitant increase of p21 expression, whereas levels of p53 were modestly affected (Fig. 1B). As expected, HEXIM1 expression was induced by HMBA, whereas did not produce significant differences in the expression of the core P-TEFb subunits (CDK9 and CycT1). Moreover, accordingly with previous studies,¹² HMBA activated expression from the endogenous HIV-LTR (data not shown). Because HEXIM1 together with 7SK RNA sequesters the core active P-TEFb in an inactive 7SK-HEXIM1/P-TEFb complex we examined the association of HEXIM1 to P-TEFb by co-immunoprecipitation. Cell extracts prepared from huHL6 cells were immunoprecipitated with anti-CDK9 and the presence of the associated CycT1 and HEXIM1 was evaluated by western blotting. HMBA treatment for 16 or 24 hr did not result in detectable differences in CDK9-associated proteins^{9,13} and data not shown. In contrast, cell extracts prepared after a short treatment with HMBA (1, 2 and 4 hr) caused a significant reduction of HEXIM1 associated with the immunoprecipitated CDK9 (Fig. 1C). Notably, the CDK9-CycT1 complex was unaffected. Thus, HMBA treatment specifically induces a rapid and transient dissociation of HEXIM1/P-TEFb complex, without altering the CDK9/CycT1 heterodimer stability.

Next we investigated a possible change in CDK9 kinase activity after HMBA treatment for short period of time. P-TEFb activity was assayed using as substrate the CTD4 peptide consisting of four consensus repeats of the RNAPII CTD, and for an accurate comparison time-course kinase assays were performed as previously described.^{4,6,9} Briefly, P-TEFb and its associated factors were affinity purified with anti-CDK9 antibody from cell extracts at various time after treatment. Immunoprecipitates were analyzed by immunoblotting with

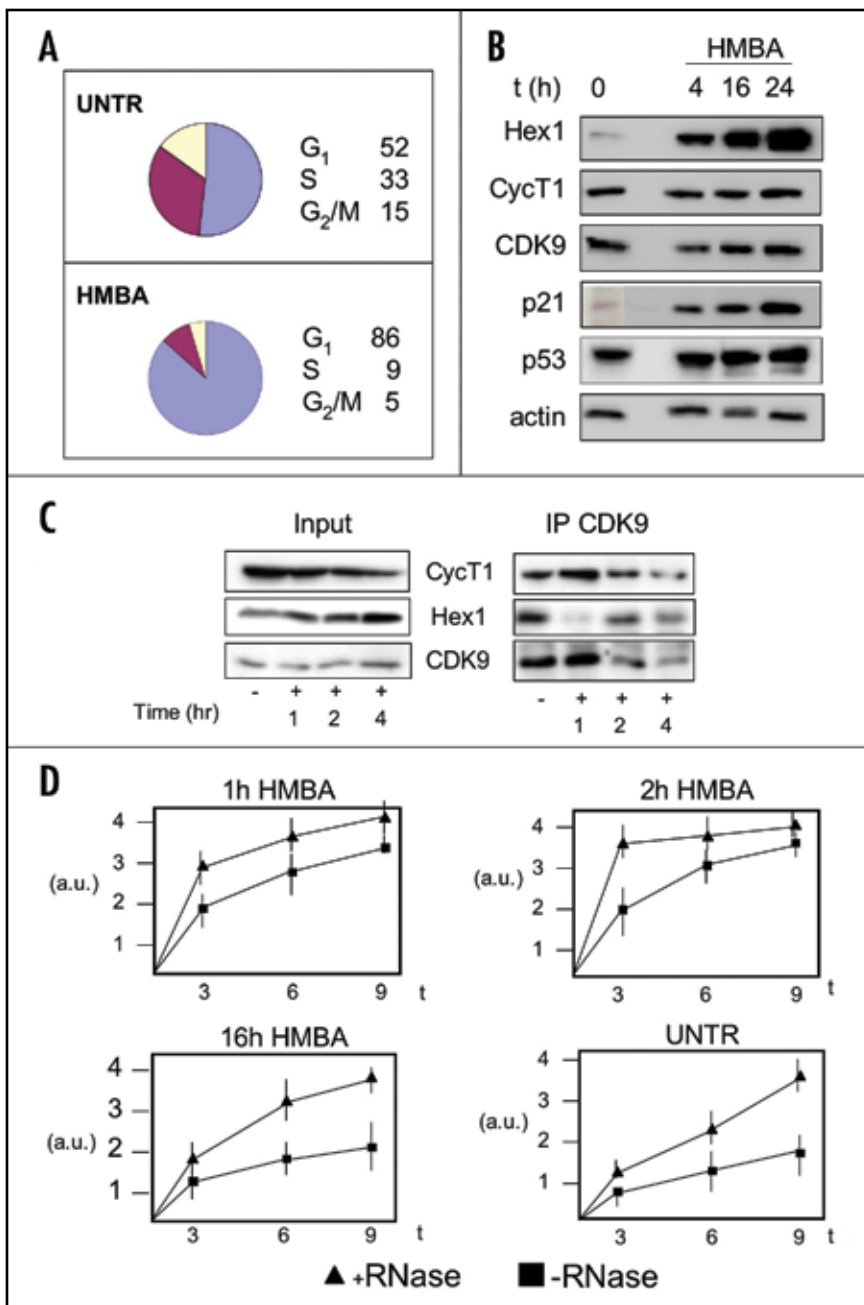


Figure 1. (A) Cell cycle distribution of huHL6 cells treated with HMBA (10mM) for 24 hr, using propidium iodide staining. (B) huHL6 cell extracts were prepared after HMBA treatments for indicated times (hr) and analyzed by Western blots with the indicated antibodies. (C) huHL6 cell extracts were prepared from cells treated with HMBA for the indicated time periods (hr), and immunoprecipitated with anti-CDK9 and analyzed by Western blots with the indicated antibodies. Panel D, huHL6 cells were treated with HMBA and after 1, 2 and 16 h of treatments, cell extracts were prepared and immunoprecipitated with anti-CDK9. The relative amounts of immunoprecipitated CDK9 were quantitated by immunoblotting. Kinase assays were performed using the CTD4 peptide and ³²P incorporation was quantified in arbitrary units and plotted versus time (min). Samples were treated or not treated with RNase, as indicated. This experiment was performed three times with similar results.

anti-CDK9 antibody for evaluation of CDK9 protein. The immunoprecipitates were then treated or not treated with RNase A. As shown in Fig. 1D, an increase in CDK9 kinase activity was observed in the samples prepared from HMBA-treated cells. Increased kinase activity was only seen in samples derived from short period of

HMBA treatment (1 and 2 hr). Moreover, the kinase activities of samples that were treated with RNase were quantitatively the same at all time points indicating that the bulk of P-TEFb present in the cell during treatment remained constant. Altogether, these results demonstrate that HMBA leads to a rapid and transient dissociation of HEXIM1 from P-TEFb with a concomitant enhancement of CDK9 kinase activity.

As presented above HMBA treatment leads to transient enhancement of P-TEFb activity with a concomitant activation of p21 expression. Because, it is well documented the activation of the p53-p21 axis in response to a variety of stimuli leading to cell cycle arrest, we investigated whether p53 might mediate the P-TEFb recruitment at p21. A prerequisite for such hypothesis is the interaction between p53 and P-TEFb. To test such premise, p53^{-/-} H1299 cells were transiently transfected with CMV-p53 and CMV-CycT1 expression vectors and cell extracts were prepared and subjected to CoIP with anti-CycT1 or anti-p53, respectively. As reported in Fig. 2A, in addition to the expected CDK9 and HEXIM1 proteins, p53 was found in the immunoprecipitated CycT1 materials. Reciprocally, anti-p53 antibody coprecipitated CycT1 and CDK9 proteins, however, no HEXIM1 protein was detectable in the p53-containing complex. Next, to ascertain whether endogenous p53 interacts with P-TEFb, CoIP analysis was carried out with cell extracts from p53-expressing U2OS cells. In Figure 2B, anti-p53 antibody coprecipitated CycT1 and CDK9, but preimmune serum did not. Reciprocally, anti-CycT1 coprecipitated endogenous p53, as well as the expected partners CDK9 and HEXIM1, whereas control IgG did not. The absence of HEXIM1 protein in p53-associated materials suggest that p53 interacts only with the catalytic active P-TEFb 'core' complex.

The foregoing observations suggest that HMBA-activation of p21 expression involves P-TEFb. Thus, we sought to investigate the presence of P-TEFb at the target promoter using chromatin immunoprecipitation (ChIP) (Fig. 2B). After treatment of huHL6 cells with HMBA for 10h, chromatin was prepared and subjected to ChIP assays with antibodies specific for CDK9, and p53. Precipitated DNA was analyzed by PCR with primers spanning four p21 sequences¹³ i.e., the high-affinity p53-binding site (-2283), two regions proximal to the transcription start site (-20 and +182) and the distal amplicon at +5794. The semiquantitative nature of these assays was taking in account by performing PCR amplification using serial dilutions of DNA template as well as by repeating the experiments (3–4

times) using different chromatin preparations. Normal serum and input DNA values were used to subtract/normalize the values from ChIP samples. ChIPs from treated cells reveal p53 recruitment at the major p53-binding site (-2283), while p53 recruitment increases only modestly at proximal core promoter (-20), and it was absent

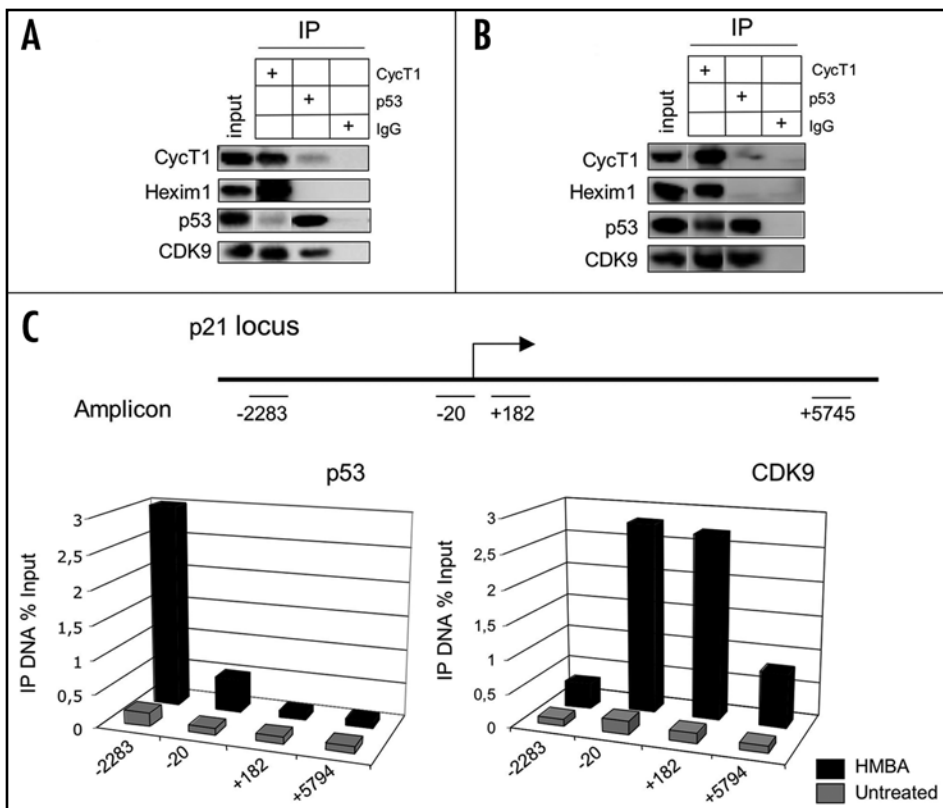


Figure 2. (A) Cell extracts from H1299 cells transiently transfected with p53 and CyclinT1 constructs were immunoprecipitated with anti-p53 or anti-CycT1, as indicated, and inputs and precipitates (IP) were analyzed by immunoblotting with the indicated antibodies. (B) U2OS cellular extracts were precipitated with anti-p53 or anti-CycT1, as indicated; and inputs and precipitates (IP) were analyzed by immunoblotting with the indicated antibodies. Panel C top, schematic representation of the p21 amplicons used in the ChIP experiments. ChIP assays were performed with extracts obtained from huHL6 cells before or 10 hr after HMBA treatment with antibodies recognizing p53 and CDK9, and the relative levels of ChIP-enriched DNA are shown. Values are expressed as percentage of input DNA immunoprecipitated. The results shown are the average of at least two separate immunoprecipitations from three independent cell cultures. All standard deviations were <15%.

at the 3' portion of the gene. As compared to untreated cells, ChIP experiments indicated that CDK9 levels rise significantly around the core promoter (-20 and +182 amplicons). Moreover, accordingly with previous studies¹³ the presence of CDK9 in the distal region (+5794) is suggestive of the presence of P-TEFb throughout the active transcription unit.

Because gene transcription is the endpoint of signal transduction pathways that mediate the cellular response to different stimuli, it is not surprising that several agents that affect cellular homeostasis impinge on the P-TEFb activity. The major mechanism regulating P-TEFb is the dynamic association of the active heterodimer CDK9/CycT1 to HEXIM1 or 2 and 7SK snRNA, for a recent review (see ref. 1). HEXIM1 was originally isolated as a protein up-regulated by HMBA, which is a potent inducer of growth arrest of a wide variety of transformed cells, and it consistently induces murine erythroleukemia cells to terminal erythroid differentiation and cell cycle arrest.^{9,10}

Despite the overall increase in HEXIM1 protein level, HMBA induces a transient dissociation of P-TEFb from the negative HEXIM1/P-TEFb complex leading to enhanced kinase activity of CDK9. Notably, such effects were not restricted to HeLa cells as similar responses were seen using 293T and MEL cells (data not

shown). While this work was in progress we learned that HMBA-mediated reversible disruption of the HEXIM1/P-TEFb complex was shown by another laboratory;¹² although, only the association of HEXIM1 to P-TEFb has been monitored in this study. Moreover, in this report,¹² only a minor effect on cell proliferation was observed, while we found cell growth arrest in response to HMBA treatment of different cell lines (HeLa, 293T, H1299, MEL, U2OS data not shown). We don't know how to explain the discrepancy between these data; however, both sets of experiments confirmed the reversible disruption of the HEXIM/P-TEFb interaction.

We presented evidences showing that HMBA treatment induces activation of p21 and recruitment of P-TEFb to the p21 locus. It is not surprising that P-TEFb is recruited to the p21 locus as it has been demonstrated that p21 is regulated at post-initiation step.¹³ How P-TEFb is recruited to p21 locus is an important issue. Because HMBA does not inhibit HDACs or induce histone hyperacetylation,¹⁰ it is unlikely a role of Brd4 in recruiting P-TEFb. Accordingly, we found that exposure to HMBA does not induce hyperacetylation at the p21 as well as at HIV-LTR promoter (data not shown). The major molecular response to HMBA exposure is the rapid increase of the catalytic activity of the P-TEFb. The finding that P-TEFb interacts with p53 makes a coherent argument on the role of this master p21-regulator in mediating P-TEFb recruitment. Moreover, it has recently been shown that CDK9 phosphorylates p53 in vitro on Ser

33, 315 and 392, although the biological role of this phosphorylation remains to be elucidated.^{14,15}

The biochemical consequences of HMBA treatment are reminiscent of the rapid release of active P-TEFb complex after treatment with stress-inducing agents such as UV irradiation and with general inhibitor of transcription as Actinomycin D or DRB.^{2,3} It is conceivable that increased availability of active core P-TEFb will help the execution of the transcriptional response leading to cell cycle arrest through activation of selected target genes.

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